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ANALYSIS OF FACTORS DETERMINING YIELD IN CROP PLANTS. I. VARIETAL DIFFERENCES IN YIELD OF GRAIN AND STRAW OF WHEAT AS INFLUENCED BY PHOTOPERIODIC TREATMENTS[1]

K. K. NANDA[2] AND J. J. CHINOY

DEPARTMENT OF BOTANY, UNIVERSITY OF DELHI, DELHI 8, INDIA

It has been shown that grain yield in wheat and other cereals is determined by a number of growth characters such as plant height, number of fruitful tillers and various ear characters which in their turn are profoundly influenced by external environment (1, 2, 3, 4, 5, 6, 13, 14, 15, 16, 17, 18, 19, 20, 21, 24, 25, 26, 27, 29, 30, 32, 33, 35, 36, 37, 38, 39, 40, 41). The purpose of this paper is to analyze the various growth and environmental factors that influence the ultimate yield of wheat. A simple and convenient way of studying the effect of environment is to subject the plants to differential photoperiodic treatments. It has already been shown (10) that the photoperiodic treatment markedly affects the vegetative period of a variety. When these changes in the vegetative periods are brought about by photoperiodic treatments, concurrent changes in response to temperature and other environmental conditions take place during some of the critical stages of plant development. Studies reported here were undertaken with a view to elucidating the influence of photoperiodic responses of plants on the interrelation of yield characters, yield and environmental factors. It is hoped that such studies will elucidate the causes of variation in yields of crop plants from year to year.

EXPERIMENTAL PROCEDURE

Three pure line varieties of wheat (*Triticum vulgare* vars. N.P. 165; N.P. 52 and P.C. 591) were used for these experiments. Graded seeds were germinated in Petri-dishes on a thin layer of sterilized sand and were exposed immediately to the following photoperiodic treatments: 1. complete darkness; 2. six-hour daily illumination with normal daylight alternating with 18 hours of darkness; 3. almost equal periods of light and darkness (normal daylight of October–March at Delhi); 4. eighteen hours of daily illumination alternating with six hours of darkness (normal daylight supplemented by artificial illumination from four 200-watt daylight bulbs at a mean distance of 2 ft from the seed for a requisite period; 5. continuous illumination (normal daylight supplemented at night with artificial illumination as in 4).

After the completion of these photoperiodic treatments which have been called "seedling" treatments, the seedlings were transplanted and were then subjected to (a) a short day (SD) with six hours of illumination, (b) normal day (ND) with normal illumination of October–March at Delhi, (c) long day (LD) either 18 hours or 24 hours of illumination as shown for different experiments. These treatments have been referred as "post-seedling" treatments. Different combinations of seedling and post-seedling treatments are described in the text by combining the serial number of the former with the serial number of the latter. Thus, for instance, 3C means that the seedlings received almost equal periods of light and darkness during the seedling stage and long day during the post-seedling stage. Further experimental details are given below separately for each experiment.

EXPERIMENT 1, *Varieties Used:* Three—N.P. 165, N.P. 52 and P.C. 591.

Photoperiodic Treatments: Petri dishes containing 25 graded seeds falling within a range 45 to 50 mg were moistened with 5 ml of water and placed under the five seedling treatments for 12 days. Twenty-seven Petri dishes (9 for each of the 3 varieties) were placed under each treatment. Three post-seedling treatments were started immediately after the transplantation of seedlings to pots.

EXPERIMENT 2: In order to confirm the results obtained, experiment 1 was repeated the following year with some modifications.

Photoperiodic Treatments: Only three seedling treatments, nos. 1, 3 and 5, were used in this experiment. Duration of treatment was increased to 14 days. In the post-seedling treatments, while short day and normal day treatments were the same as in experiment 1, long day treatment was continuous light instead of 18-hour day, as in experiment 1. In all, 81 Petri dishes with 25 seeds in each were exposed to different seedling treatments (27 for each variety).

EXPERIMENT 3, *Variety Used:* One—N.P. 52.

Photoperiodic Treatments: The photoperiodic treatments were the same as in experiment 2. After

[1] Received February 9, 1956.

[2] Currently working in the Department of Botany, University of California, Los Angeles, as Junior Research Botanist. Came to the United States as a post-doctoral visiting fellow under the Fulbright International Educational Exchange Program of the U. S. Department of State.

the completion of the seedling treatments, the seedlings were transplanted in rows in small plots prepared for the purpose in the field. The nature of the experiment imposed certain limitations on the layout of the experiment. As long day treatment during the post-seedling stage was likely to affect the results of the other two treatments, if kept in proximity the LD plot was some distance away from the other two. Thus three blocks were made for the three post-seedling treatments and three replications for each treatment combination were randomized together with the seedling treatments in each block (30′ × 15′). The randomization within each block was done in order to eliminate any errors arising from differences in the intensity of illumination at various points in the block. The error due to soil heterogeneity was eliminated as far as possible by adequate manuring and watering. The main purpose of doing this experiment in the soil was to remove restriction to root growth which is generally brought about by the limited space available in the pots.

Each plot contained 5 rows, 6 inches apart with about 25 seedlings in each row (4″ apart). After transplantation fertilizer was provided thrice at fortnightly intervals at the rate of 7.50 gm of superphosphate and 3.75 gm of potassium sulphate per plot. Further details of experimental procedure have been outlined elsewhere (10).

Observations: After the plants were fully mature, they were harvested and data were collected for the various yield characters, viz., length and number of ears, number of spikelets and grains per ear, thousand grain weight and finally for the grain and straw yields. The present paper deals only with the results of grain and straw yields. The grain was weighed separately for each treatment while the rest of the dry matter of the plant including the husk was taken as straw weight. The results have been expressed in grams per plant.

Experimental Findings

The results of grain and straw yields per plant are presented diagrammatically in figure 1. The effects of various combinations of "seedling" and "post-seedling" treatments are shown in this figure separately for each variety in all the three experiments. In order to bring out the effects of "seedling" and "post-seedling" treatments as well as varieties, the data of grain and straw yields have been condensed by adding all the values of any one level of the factor and dividing by the total number of observations. These treatments are described in the text by their respective serial numbers presented within brackets, and the values given are the averages obtained by condensing the data as explained here. Thus, for example, the effect of long-day treatment during the post-seedling stage on grain yield was obtained by adding all the values of grain yield under this treatment irrespective of the seedling treatment and variety and dividing the sum by 15 (5 seedling treatments × 3 varieties), and this effect is referred as as (c). The single factor effects obtained by compiling the data in this manner are shown in table I. It will be seen from this table that the grain and straw yields are markedly reduced both under short and long "post-seedling" treatments. Effects of seedling treatments are not as pronounced as those of post-seedling treatments, probably because of the shorter duration of treatment. It is, however, evident that in experiment 1 both the grain and straw yields increase from treatments 1 to 4 and then decrease in 5. In experiments 2 and 3, in which treatments 2 and 4 were not included, the yields under treatments 1 and 5 are lower than those under treatment 3 except for the grain yield in treatment 5 of experiment 2. The varietal effects in general show a lower grain yield in P.C. 591 as compared with the other two varieties, which do not appear to differ from each other in their yields (table I).

Analysis of variance was carried out separately for grain and straw yields of all the three experiments. Details of the analysis have not been included here. The significance or otherwise of the single factor effects and their interactions have been shown in table II. (S denotes a highly significant result ($P < 0.01$); s denotes significance at $P < 0.05$, and results which are not significant are denoted by an "x.")

It can be seen from table II that the effects of "post-seedling" treatments are highly significant in all the experiments. Of the "seedling" treatments, while grain and straw yields in experiment 1 are highly significant, in experiment 2, the significance is at 5% level and in experiment 3 the results are not signifi-

Table I

Single Factor Effects—Grain and Straw Yields

Treatments and vars.	Gm/plant					
	Expt 1		Expt 2		Expt 3	
	Grain	Straw	Grain	Straw	Grain	Straw
Seedling treatments *						
1	0.868	1.913	1.056	1.968	1.895	3.984
2	1.253	2.260	....	....	....	....
3	1.493	2.866	1.399	2.580	2.416	4.235
4	2.155	3.954	....	....	....	....
5	1.428	2.841	1.480	2.139	2.138	3.615
Post-seedling treatments **						
a	0.522	1.237	0.932	1.528	0.837	1.684
b	2.903	5.059	2.440	3.579	4.537	7.271
c	0.656	2.063	0.550	1.581	1.075	2.880
Varieties †						
N.P. 165	1.377	2.806	1.439	2.551	....	....
N.P. 52	1.419	2.635	1.450	2.210	....	....
P.C. 591	1.285	2.919	0.999	1.926	....	....

* Seedling treatments: 1, complete darkness; 2, normal daylight 6 hrs alternating with 18 hrs darkness; 3, nearly equal periods of light and darkness; 4, daily illumination 18 hrs alternating with 6 hrs darkness; 5, continuous illumination.

** Post-seedling treatments: a, short day (SD); b, normal day (ND); and c, long day (LD).

† Three pure line varieties of wheat (*Triticum vulgare*) were used.

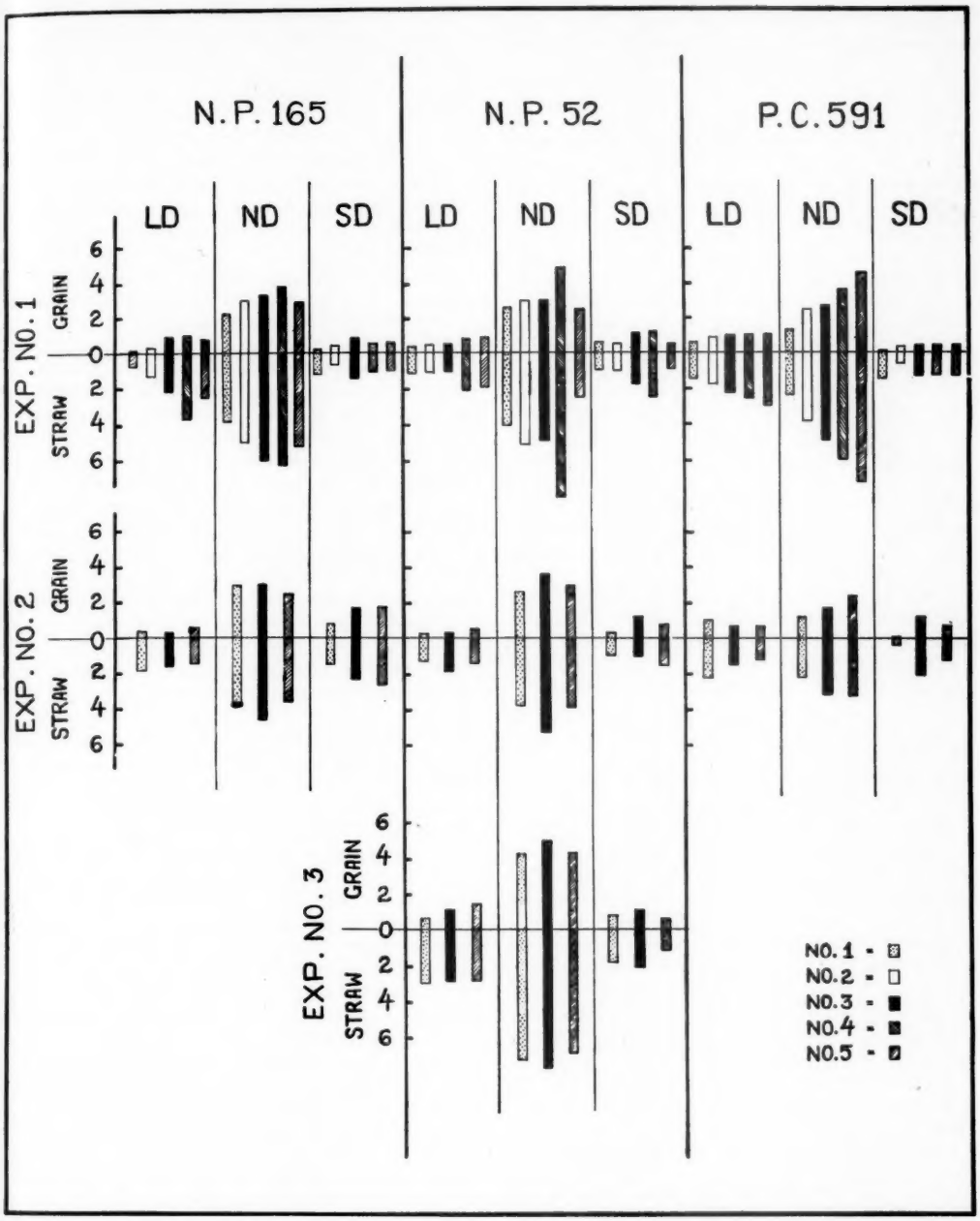

FIG. 1. Grain and straw yield (gm) in three varieties of wheat (NP. 165, N.P. 52 and Punjab C. 591) under different combinations of "seedling" and "post-seedling" treatments. Seedling treatments: 1 = Complete darkness; 2 = 6 hours daily illumination; 3 = normal day; 4 = 18 hours daily illumination and 5 = continuous illumination. Post-seedling treatment: LD = Long Day; ND = Normal Day; and SD = Short Day.

cant. The varietal differences in grain yield are significant at 5 % level and straw yield is significant only in experiment 2.

Considering the interactions (table II) it will be seen that most of the first order and even second order interactions in experiment 1 are significant. In

TABLE II

SIGNIFICANCE OF TREATMENTS AND THEIR INTERACTIONS—GRAIN AND STRAW YIELDS

TREATMENTS	EXPT 1		EXPT 2		EXPT 3	
	GRAIN	STRAW	GRAIN	STRAW	GRAIN	STRAW
Post-seedling	S	S	S	S	S	S
Seedling	S	S	s	s	x	x
Variety	s	x	s	S	..	..
Post-seedling × seedling	S	S	x	s	x	x
Post-seedling × variety	S	S	S	S	..	..
Seedling × variety	S	S	x	x	..	..
Post-seedling × seedling × variety	S	S	x	x	..	..

S = highly significant effect ($P < 0.01$).
s = significant effect ($P < 0.05$).
x = results not significant.

experiment 2, interaction seedling treatment × variety is statistically not significant both for grain and straw yields. The second order interaction is also not significant in this case.

It will be seen from figure 1 that in experiments 1 and 2 the seedling treatment that gives optimum yield varies with the variety. Thus, for instance, with N.P. 165 and N.P. 52, treatment 4 is most beneficial, while with P.C. 591, the optimum effect is shown by plants of treatment 5. It is interesting to note further that the beneficial effect of treatment 4 is more marked in N.P. 52 than in N.P. 165. It has been shown previously (10) that N.P. 165 is the earliest and P.C. 591 the latest flowering of these three varieties. This suggests that there is a relationship between the vegetative period of variety (mean period between the date of sowing and anthesis—10) and its photoperiodic response. The later the flowering of a variety, the greater is the beneficial effect of the long-day in the seedling treatment. Thus, treatment 5 which adversely affects the grain and straw yields of N.P. 165 and N.P. 52, which are early varieties, favorably influences the yields in P.C. 591 which is a comparatively later flowering variety (10). Similarly treatment 4 which enhances the yield of N.P. 52 has only a slight effect on N.P. 165. The same conclusions can be drawn as regards the interaction between post-seedling treatment and variety both for grain and straw yields. Thus LD in the post-seedling treatment reduces grain and straw yields to a far greater extent in N.P. 165 and N.P. 52 than in P.C. 591. Under ND treatment N.P. 165 is the highest yielder and P.C. 591 the poorest. N.P. 52 does not vary significantly from N.P. 165. Under SD treatment also P.C. 591 yields much lower than N.P. 165 or N.P. 52.

Comparing the combined effects of seedling and post-seedling treatments, it will be clearly seen that the effect of the post-seedling treatment is predominant, and seedling treatment has a slight modifying effect (fig 1).

GENERAL DISCUSSION

In elucidating effects of seedling and post-seedling treatments on yields of grain and straw the direct as well as the indirect influences of light are to be considered. A low assimilatory activity, as already seen in a previous communication (11, table 8), is responsible for reduction in the vegetative growth of SD plants because of considerable losses by respiration during the long dark period of each day, in addition to the very low production of carbohydrates. This direct effect also appears to be the main reason for reduction in straw and grain yields of SD plants in all the varieties. This is confirmed from the results of correlation coefficients between grain yields and net assimilation rates (daily increase in dry matter in gm per gm of leaf dry weight) and straw yields and net assimilation rates (grain: $r = 0.779$ and straw: $r = 0.819$). In the calculation of these correlation coefficients net assimilation rates and grain yields of LD plants have been omitted, because the lower yields in the long-day treatment are not due to the low assimilatory activity as seen from net assimilation rates of these plants (11, table 8). This is further substantiated by working out the correlation coefficients between net assimilation rates of LD-ND plants and their straw and grain yields. Highly significant inverse correlations are obtained in this case ($r = -0.939$ for straw, and $r = -0.961$ for grain). This clearly indicates that the factor which causes a reduction in yields of straw and grain under long days is not the deficiency of carbohydrates but some other factor. Considering other nutritional factors such as uptake of N, P and K it also appears that the reduction in yields of straw and grain by short days is due to the very slow uptake of these nutrients (12). It is, therefore, clear from the above mentioned facts that the effect of light on yields of straw and grain is a direct one in the case of short days resulting from its low assimilatory activity, while on the other hand, it is an indirect one in the case of long days, operating through regulatory mechanism of its development process (12). In normal days (ND) there is a balanced daily photoperiod and dark period for the varieties under experimentation and, therefore, sufficient vegetative growth takes place leading to a higher grain yield compared to the other two treatments. A reduction in the grain yield by short and long day treatments has also been reported by Hurd Karrer (22, 23), Maximov (28) and Rasumov (34).

The increase in grain yield of P.C. 591 under the "seedling" treatment 5 may be partly due to the earlier ripening of the variety and the consequent lower temperatures during ripening. It has already been shown by one of us that the temperature of the ripening period has a profound effect upon the grain filling processes in wheat and consequently upon its yield (7, 8). The lowering of grain yield under SD treatment may not only be due to the reduction in the vegetative growth as a result of the low assimilatory activity of the plant, but also may be due to the shifting of the ripening period of plants under this treatment in a higher range of temperature which is

TABLE III

SINGLE FACTOR EFFECTS—GRAIN/STRAW RATIO

TREATMENTS AND VARS.	GRAIN/STRAW RATIO		
	EXPT 1	EXPT 2	EXPT 3
	Seedling treatments *		
1	0.454	0.536	0.482
2	0.554	....	....
3	0.521	0.542	0.576
4	0.545	....	....
5	0.503	0.692	0.588
	Post-seedling treatments **		
a	0.422	0.609	0.292
b	0.574	0.682	0.626
c	0.318	0.348	0.665
	Varieties †		
N.P. 165	0.491	0.564	....
N.P. 52	0.538	0.656	....
P.C. 591	0.440	0.518	....

*, **, † See table I.

less favorable for ripening of grain. This is confirmed by the shortening of the ripening period of SD plants (unpublished data). This shifting of ripening of grain in a higher range of temperature may cause a hastening of cell division and other processes leading to the quicker ripening of the grain as already shown (7). That the lowering of grain yield in SD treatment is not entirely due to a lower assimilatory activity, but may partly be due to the adverse effect of the temperature of the ripening period is also indicated by the results of grain/straw ratios presented in table III. Generally speaking the ratios are lower for SD and LD plants. This tendency for greater vegetative growth is even observed for some of the seedling treatments, especially for treatment 1 (table III). The lower grain yields in SD and LD treatments may also be partly due to the sterility induced by these light treatments as shown previously (9, 31). But even after taking into account the sterility factor some part of the over-vegetativeness can be ascribed to the reduction in weight of the grain of SD plants, due to its ripening in a higher range of temperature. In order to elucidate the effects of temperature, it would be necessary to eliminate the effects of other factors such as the number of ears, number of grains per ear, number of spikelets per ear and so on. A full analysis of all these factors affecting yield under different photoperiodic treatments will be attempted in subsequent communications.

SUMMARY

1. The present paper records the results of the investigation on the effect of seedling and post-seedling treatments on grain and straw yields of three varieties of wheat (*Triticum vulgare* vars. N.P. 165, N.P. 52 and P.C. 591).

2. Grain and straw yields are markedly reduced under both SD and LD treatments. The reasons for the lowering of yields under the two light treatments are, however, quite different. In SD treatment the reduction is directly due to the low rate of assimilation as is evident from the highly significant correlation between yields and net assimilation rates. In the case of LD treatment on the other hand, the effect appears to be due to the influence of light on the formation of regulatory substances.

3. Analysis of variance reveals that most of single factor effects are significant (table I). Few of the interactions are also significant (table II). Thus for instance, seedling treatment 5 which results in a reduction in yields of N.P. 165 and N.P. 52, increases the yield of P.C. 591, which is a late flowering variety.

4. This increase in the grain yield of P.C. 591 under the photoinductive treatment 5 is considered to be partly due to the shifting of the ripening period of the variety in a range of temperature which is more favorable for the grain filling processes.

5. Grain/straw ratio is also lower under SD and LD treatments (table III). This clearly indicates that the lowering in grain yield under SD treatment is not entirely due to a lower assimilatory activity but may also be partly due to the adverse effect of the higher temperature of the ripening period in these plants.

We wish to thank Professor Karl C. Hamner, Chairman of the Department of Botany, University of California, Los Angeles for critical reading of the papers reported in this series of communications and for his helpful suggestions.

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ANALYSIS OF FACTORS DETERMINING YIELD IN CROP PLANTS. II. THE INFLUENCE OF PHOTOPERIODIC TREATMENTS ON CHARACTERS DETERMINING YIELD IN WHEAT WITH SPECIAL REFERENCE TO THE TEMPERATURE OF THE RIPENING PERIOD[1]

K. K. NANDA AND J. J. CHINOY

DEPARTMENT OF BOTANY, UNIVERSITY OF DELHI, DELHI-8, INDIA

The previous paper (23) in this series dealt with the varietal differences in the yield of grain and straw of wheat under varying photoperiodic treatments given during the seedling and post-seedling stages. It was shown there that the lowering of grain and straw yield under SD-photoperiod was not entirely due to the lower assimilatory activity of plants under this light treatment. It was also shown that reduction in yields of grain and straw of plants under LD-photoperiod took place in spite of its higher rate of net assimilation (11). It was suggested that one of the contributory factors responsible for the lowering in yield was most probably the higher temperature of the ripening period. In order to evaluate the effect of temperature of the ripening period in the present case, the effect of other yield characters as influenced by differential photoperiodic treatments will also have to be taken into consideration. An analysis of these yield characters and the effect of light and temperature on these have been undertaken in this paper.

EXPERIMENTAL PROCEDURE

For details of experimental procedure, a reference may be made to the earlier paper (23). When plants had reached the full-ripe stage, they were harvested. Each plant was uprooted separately and records were taken for the following yield characters for ten plants under each treatment:

1. Number of ears per plant,
2. Length of ear,
3. Number of spikelets per ear,
4. Number of grains per ear, and
5. Thousand grain weight.

EXPERIMENTAL FINDINGS

As the data for the various characters determining yield are very extensive, results of only one experiment (expt 1) have been presented here in figure 1. Those of experiments 2 and 3 follow more or less similar trends and only the significance of the treatments and their interactions in these experiments are presented in table II along with those of experiment 1. The details of the analysis of variance have not been included in this table. Single factor effects of light treatments and varieties in experiment 1 were also obtained by compiling the data as explained previously (23) and are given in table I.

The number of ears per plant under different combinations of seedling and post-seedling treatments are shown by histograms in the first row of figure 1.

TABLE I

SINGLE FACTOR EFFECTS (EXPT 1)

TREATMENTS AND VARS.	NO. EARS PER PLANT	LENGTH OF EAR (CM)	NO. SPIKELETS PER EAR	NO. GRAINS PER EAR	THOUSAND GRAIN WT
		Seedling treatments *			
1	1.88	6.83	15.2	14.8	27.1
2	2.07	7.19	15.9	17.9	29.5
3	2.37	7.89	17.2	18.7	31.5
4	3.27	7.79	17.3	16.8	30.2
5	2.78	7.20	16.9	14.6	30.9
		Post-seedling treatments **			
a	1.51	7.29	16.7	13.9	23.2
b	2.97	8.26	18.1	28.3	35.6
c	2.97	6.59	14.6	7.4	30.8
		Variety †			
N.P. 165	2.54	8.27	16.1	15.9	29.4
N.P. 52	2.66	6.39	16.6	18.1	27.0
P.C. 591	2.21	7.47	16.9	15.6	33.1

* Seedling treatments: 1, complete darkness; 2, normal daylight 6 hrs alternating with 18 hrs darkness; 3, nearly equal periods of light and darkness; 4, daily illumination 18 hrs alternating with 6 hrs darkness; 5, continuous illumination.

** Post-seedling treatments: a, short day (SD); b, normal day (ND) and c, long day (LD).

† Three pure line varieties of wheat (*Triticum vulgare*) were used.

It is seen that the number of ears decreases both under SD and LD post-seedling treatments. Reduction in the number of ears is also noticeable in seedling treatments 1 and 2. Treatments 4 and 5, however, appear to increase the number of ears. This is clearly brought out from table I which gives the mean effects of the main treatments and varieties obtained by compiling the data as explained previously (23). It is seen that the number of ears increases from seedling treatments 1 to 4 and then decreases in treatment 5, though the number is still higher than that in treatment 3. Results of analysis of variance show that the treatments and their interactions are all significant in experiments 1 and 3. In experiment 2, however, the interactions between variety and seedling and between variety and post-seedling treatments are significant only at the 5 % level (table II).

Results of the length of ear are shown in the second row of figure 1. It is seen that the length of ear is very much reduced under all combinations of seedling treatments with SD and LD post-seedling treatments. Thus the length of ear is reduced even in treatments 4 and 5 which increase the number of ears

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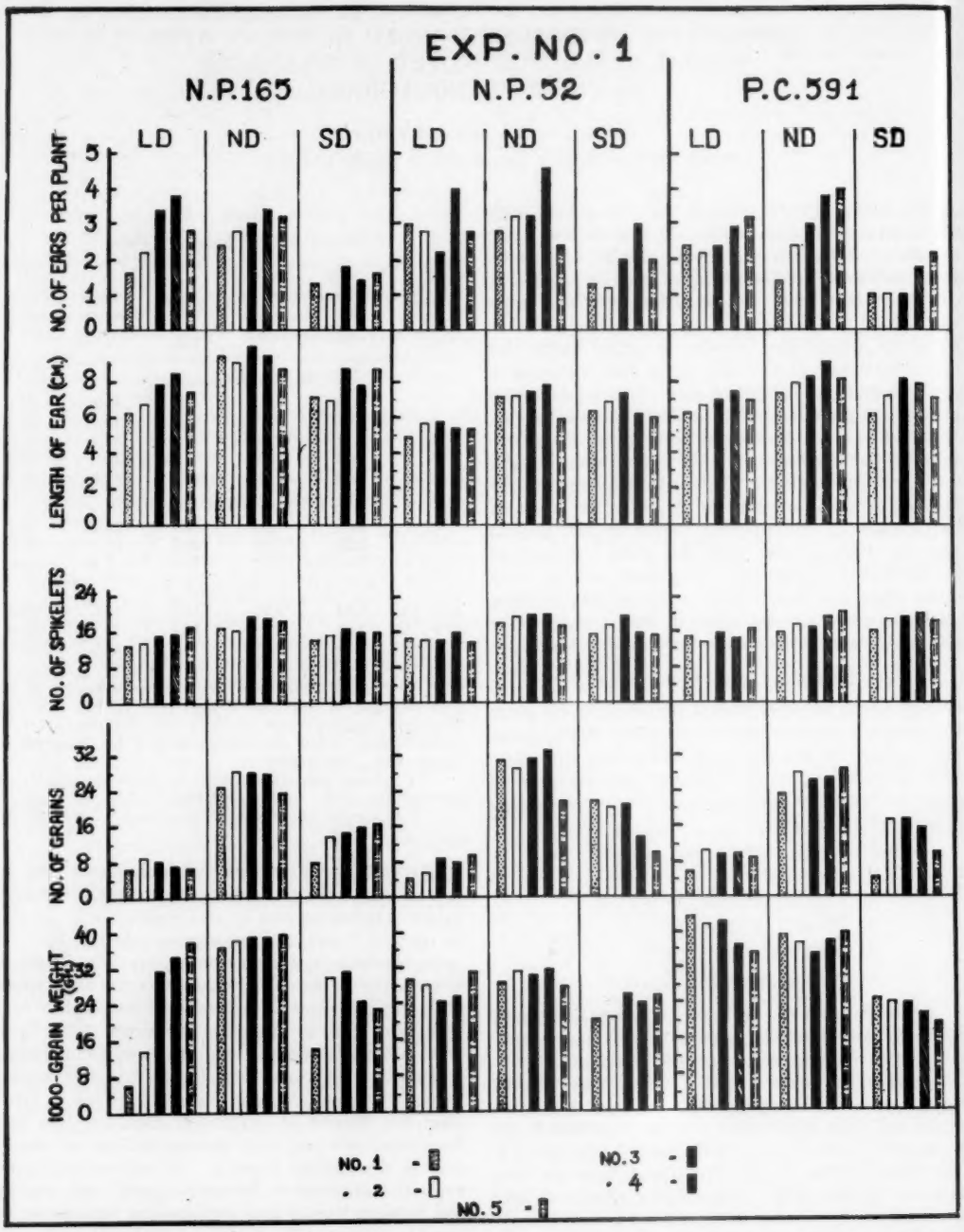

FIG. 1. Effect of different combinations of seedling and post-seedling treatments on various characters determining yield in three varieties of wheat (N.P. 165, N.P. 52 and Punjab C. 591). Seedling treatments: 1—Complete darkness; 2—6 hrs daily illumination; 3—normal day; 4—18 hrs daily illumination; 5—continuous illumination. Post-seedling treatments: LD = long day; ND = normal day, and SD = short day.

TABLE II

SIGNIFICANCE OF TREATMENTS AND THEIR INTERACTIONS

TREATMENTS	NO. EARS PER PLANT			LENGTH OF EAR			NO. SPIKELETS PER EAR			NO. GRAINS PER EAR			THOUSAND GRAIN WT		
	EXPT 1	EXPT 2	EXPT 3	EXPT 1	EXPT 2	EXPT 3	EXPT 1	EXPT 2	EXPT 3	EXPT 1	EXPT 2	EXPT 3	EXPT 1	EXPT 2	EXPT 3
Post-seedling	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Seedling	S	S	S	S	S	S	S	S	S	S	S	S	s	S	x
Variety	S	S	–	S	S	–	S	x	–	S	x	–	S	S	–
Post-seedling × seedling	S	S	S	S	S	s	S	x	x	S	S	x	x	S	S
Post-seedling × variety	S	s	–	S	S	–	S	S	–	S	S	–	S	S	–
Seedling × variety	S	s	–	S	x	–	S	x	–	S	x	–	S	S	–
Post-seedling × seedling × variety	S	S	–	S	s	–	S	x	–	S	x	–	S	S	–

S = highly significant effects ($P < 0.01$).
s = significant effects ($P < 0.05$).
x = effects not significant.

per plant (table I). The effects of seedling treatments, however, are not so pronounced as those of post-seedling treatments probably because of the very short duration of the seedling treatment.

The results of the analysis of variance (table II) clearly demonstrate that all the single factor effects are highly significant in all three experiments. The interaction between post-seedling treatment and variety is also significant. Thus the length of ear is reduced by LD-photoperiods in post-seedling treatment to a much greater extent in N.P. 165, which is the earliest of the three varieties under investigation, and to the least extent in P.C. 591, which flowers a few days later (9). The interaction of the light treatment with variety is evident even in the case of short duration seedling treatment where it is observed that treatment 4, which reduced the ear length in N.P. 165 and N.P. 52, actually brings about an increased ear length in P.C. 591 (fig. 1). The highly significant effects of these interactions are clearly seen from table II. The interaction between seedling and post-seedling treatments is significant in experiments 1 and 2. The second order interaction is also significant (table II) though only at the 5 % level in experiment 2.

The trend of results for the number of spikelets per ear (row 3, fig 1) is similar to the length of the ear. Thus the number of spikelets decreases both under SD- and LD-photoperiods in post-seedling treatments. This effect is shown even by the seedling treatment which is of a short duration (table I).

The results of the analysis of variance given in table II show that the effects of seedling and post-seedling treatments are highly significant in all three experiments. The varietal effects are not significant in experiment 2. Of the interactions only the post-seedling treatment × variety is significant in experiment 2, all other interactions lacking significance. In experiment 1, however, the interactions are all significant. In experiment 3, the interaction between post-seedling and seedling treatments is not significant (table II).

The general trend of results of the number of grains per ear is similar to those of the number of spikelets per ear (rows 3 and 4, fig 1). Thus the effects of seedling and post-seedling treatments as well as varieties are highly significant in experiment 1. The varietal effects are not significant in experiment 2 (table II). The interactions are also all significant except for second order interaction in experiment 2 and interaction between seedling and post-seedling treatments in experiment 3 (table II). Comparing the results of the number of grains with the number of spikelets per ear, it is worthy of note that photoperiodic treatment markedly influences the setting of grains, which is very poor in plants exposed to LD-photoperiods in post-seedling treatments. Thus in table I of experiment 1 whereas 28.3 grains are formed from 18.1 spikelets under ND treatment, only 7.4 grains are set from 14.6 spikelets under LD treatment. In other words, LD treatment results in some sort of sterility. This point will be discussed in greater detail later.

Thousand grain weights under different combinations of seedling and post-seedling treatments are represented in the last row of figure 1. The effects of the main treatments can also be seen from table I. It is clear from these that the thousand grain weight, like other yield characters, is lowered both under SD- and LD-post-seeding treatments. The differences under seedling treatments are not significant in experiment 3, but are significant at the 5 % level in experiment 1. The interactions are all significant except the one between seedling and post-seedling treatments in experiment 1 (table II). There is a marked interaction between variety and post-seedling treatment and it is interesting to observe that the LD-post-seedling treatment lowers the thousand grain weight of N.P. 165 and N.P. 52, which are early flowering varieties. On

the other hand, it increases the thousand grain weight of P.C. 591, which is a later flowering variety (9).

This increase in the thousand grain weight of P.C. 591 by LD-treatment is probably due to the shifting of the ripening period to more favorable temperature range, caused by earliness of flowering.

Discussion of Results

It has already been shown elsewhere (4, 6) that the temperature of the ripening period has a profound effect on the grain yield of wheat. Waldron (27) has also found a high negative correlation between yield of varieties and the mean maximum temperature of July. Bayfield (1) has reported a negative correlation between grain yield of wheat and temperature during the period from heading to harvest. Some workers (2, 13, 14, 19, 21, 24) working on the influence of sowing date on yield of cereals have ascribed the reduction in the grain yield in late sowing to weather conditions. The correlation coefficient between the maximum temperature during the ripening period and grain yield per plant in this experiment was worked out from the data of grain yield presented in the previous paper (23). Mean maximum temperature of the ripening period was calculated from the meteorological records by taking the sum of the daily maximum temperatures during that period and dividing it by the number of days. The correlation turned out to be insignificant in all the experiments (correlation coefficient 14, table III) indicating that the effect of the temperature was masked by some other factors operating in these experiments. This was further supported by the fact that the correlation between temperature and thousand grain weight was also insignificant in all the experiments (correlation coefficient 15, table III). Nevertheless, a highly significant positive correlation between yield per plant and thousand grain weight (correlation coefficient 16, table III) indicated that grain yield was determined by grain filling processes. Positive correlation between the thousand grain weight and yield has also been reported by some other workers (3, 15, 17, 18, 25, 26). Immer and Stevenson (16) working with oats found a strong correlation between plumpness of grain and the yielding ability. Dunham (12), however, reported that although thousand kernel weight and grain yield were very closely associated in oats, the association was much less apparent in wheat. Locke et al (20), however, did not find thousand grain weight an important character in their studies and reported high correlation between yield and the number of grains per ear.

The above results indicated that some factors including the growth of the plant influenced the grain yield of wheat under different photoperiodic treatments. Considering in the first instance the number of tillers, it was found as stated previously (10) that tillering was restricted due to the limitation imposed on root growth by the small size of the pots as well as by SD- and LD-treatments. This reduction in the number of tillers will be reflected in the number of ears produced and ultimately in the grain yield per plant. That this is so, is shown by the very highly significant positive correlations that have been found to exist between the number of ears and number of tillers per plant (correlation coefficient 1, table III) and also between the number of ears and yield per plant (correlation coefficient 2, table III). Over and above the number of ears, the size of ear is generally determined by the stem growth (5, 7) as is seen from the highly significant positive correlation between the length of the ear and final plant height (correlation coefficient 3, table III). This influence of plant growth is not only reflected in the length of the ear, but also in the number of spikelets and the number of grains (fig 1, rows 3 and 4). All the three above mentioned characters; namely, length of ear, number of spikelets and number of grains per ear influence the yield per ear under different photoperiodic treatments as is seen from correlation coefficients 4, 5 and 6 of table III.

Having eliminated the effect of the number of ears on yield, an attempt was made to determine whether the yield per ear showed any correlation with the mean maximum temperature of the ripening period. The values for the coefficient of correlation between

Table III

Total Correlation Coefficients Showing Relationship between Growth and Environmental Factors Determining the Grain Yield in Wheat

No.	Total correlation	Observed value of r		
		Expt 1	Expt 2	Expt 3
1	No. tillers and no. ears/plant	(S) +0.749	(S) +0.809	(S) +0.697
2	No. ears/plant and yield/plant	(S) +0.563	(S) +0.769	(S) +0.783
3	Length of ear and plant ht	(S) +0.540	(S) +0.576	(S) +0.657
4	Length of ear and yield/ear	(S) +0.637	(S) +0.727	(S) +0.938
5	No. of spikelets/ear and yield/ear	(S) +0.739	(S) +0.846	(S) +0.949
6	No. of grains/ear and yield/ear	(S) +0.996	(S) +0.873	(S) +0.928
7	Temp. and yield/ear	(x) −0.037	(x) −0.065	(x) −0.224
8	Thousand grain wt and yield/ear	(S) +0.794	(S) +0.468	(S) +0.794
9	Yield/ear and fertility ratio	(S) +0.984	(S) +0.807	(x) +0.107
10	Temp. and fertility ratio	(S) −0.602	(x) −0.219	(x) −0.219
11	Thousand grain wt and temp. (SD-ND)	(S) −0.599	(S) −0.816	(S) −0.940
12	Yield/ear and temp. (SD-ND)	(S) −0.729	(S) −0.794	(S) −0.915
13	Yield/ear and thousand grain wt (SD-ND)	(S) +0.861	(S) +0.864	(S) +0.920
14	Yield/plant and mean max. temp.	(x) −0.188	(x) +0.008	(x) −0.053
15	Thousand grain wt and mean max. temp.	(x) −0.243	(x) −0.061	(x) −0.302
16	Yield/plant and thousand grain wt	(s) +0.522	(s) +0.495	(s) +0.851

For S, s, x, see table II.

these two characters are, however, not significant in all three experiments. On the other hand, a very highly significant correlation between the thousand grain weight and yield per ear (correlation coefficient 8, table III) confirms the conclusion already drawn; namely, that yield is determined to a greater or lesser extent by the grain filling processes.

This masking of the effect of temperature on yield is over and above the effects of any of the growth characters enumerated above. It appears that the length of the day may be playing a direct role in masking the effect of temperature. On a closer examination of the data of the number of spikelets and the number of grains per ear in plants under different combinations of seedling and post-seedling treatments, it is realized that the fertility of the pollen was considerably influenced by the light treatment. A reference has already been made to this fact in previous publications (8, 22). It has been shown that percentage sterility of pollen grains of three varieties of wheat is increased both under LD- and SD-photoperiods, more so in the former case. This is further corroborated by the data of fertility ratio given in table IV. The fertility ratio, calculated by dividing the number of grains per ear by the number of spikelets per ear, differs from the fertility index calculated previously (22) in that the number of florets per spikelet have not been taken into account as the data of the number of florets were not available. It is seen from this table that the fertility ratio is influenced greatly by the seedling and post-seedling treatments. Even the short duration seeding treatment considerably decreases the fertility ratio in treatments 1 and 5 as well as in post-seedling treaments, SD and LD.

Considering these wide differences in the fertility ratio in plants under different seedling and post-seedling treatments, it would appear that it may have influenced the grain yield. This is borne out by the very highly significant positive correlation between the yield per ear and fertility ratio in two experiments (correlation coefficient 9, table III). The correlation is not significant for experiment 3 probably on account of the fact that the intensity of light under LD post-seedling treatment was low while in the case of SD treatment under field conditions proper shading of the plants could not be accomplished. That the fertility ratio is mainly determined by light and not by temperature is seen from the insignificant correlation between temperature and fertility ratio (correlation coefficient 10, table III). In experiment 1, however, there appears to be significant effect of temperature on fertility ratio so the temperature effect cannot be ruled out entirely.

Considering the results of the fertility ratio (table IV) it becomes obvious that this ratio is very much lowered under LD- as well as SD-post-seedling treatments, whereas simultaneously the ripening period of plants under the LD-treatment is shifted to a lower temperature range which is not likely to adversely affect the grain filling process. In plants under SD treatment, the ripening period is shifted in a higher range of temperature which has already been shown to affect adversely the grain filling process in wheat (6). It is, therefore, quite clear why the correlations between temperature and thousand grain weight or temperature and yield per plant and even the yield per ear (correlation coefficients 14 and 15, table III) are not significant. If the yield as well as thousand grain weight data of plants under LD-treatment are not included in the calculation of correlation coefficients, the adverse effect of the shifting of the ripening period of plants under SD-treatment to a higher temperature range is clearly brought out by highly significant inverse correlations between thousand grain weight and mean maximum temperature of the ripening period of plants of SD- and ND-photoperiods (correlation coefficients 11 and 12, table III). This is further supported by the highly significant positive correlation between yield per ear and thousand grain weight of such plants. It is worthy of note that the effect of the temperature of the ripening period within a certain given range is paramount over the effect of a number of growth characters on grain yield, like length of the ear, number of spikelets and grains per ear and even the fertility ratio.

TABLE IV

EFFECT OF PHOTOPERIODIC TREATMENTS AND VARIETIES ON THE FERTILITY RATIO

TREATMENTS AND VARS.	FERTILITY RATIO		
	EXPT 1	EXPT 2	EXPR 3
Seedling treatment *			
1	0.89	1.50	2.31
2	1.08	...	...
3	1.02	1.51	2.24
4	0.96	...	...
5	0.85	1.63	2.29
Post-seedling treatment **			
a	0.84	1.37	2.33
b	1.51	1.98	2.24
c	0.52	1.18	2.28
Variety †			
N.P. 165	0.95	1.53	...
N.P. 52	1.02	1.53	...
P.C. 591	0.89	1.35	...

*, **, † See table 1.

SUMMARY

1. The present paper deals with the results of the investigations on the effect of seedling and post-seedling photoperiodic treatments on various characters determining grain yield; namely, the number of ears per plant, the length of the ear, the number of spikelets and grains per ear and the thousand grain weight in three varieties of wheat (*Triticum vulgare*, vars. N.P. 165, N.P. 52 and P.C. 591).

2. The number of ears per plant, the length of the ear, the number of spikelets and grains per ear and the thousand grain weight are all reduced under both SD- and LD-post-seedling treatments.

3. The seedling treatments also affect the yield characters, though the effect is not so pronounced.

4. The results of analysis of variance reveal that the single factor effects are practically all significant. Of the first order interactions, those between seedling treatment and variety and between post-seedling treatment and variety are also significant in all cases.

5. Correlation coefficients between grain yield and various growth and environmental factors have been worked out and discussed with a view to analyzing the causes for the masking of the effect of temperature of the ripening period on the grain yield; which effect is found to lack significance in these experiments.

6. Highly significant positive correlations exist between the grain yield and the various yield characters and also between these yield characters and growth characters. This indicates that the masking of the effect of the temperature of the ripening period in these experiments is brought about by growth characters; namely, the number of tillers and plant height which are reduced under LD- and SD-post-seedling treatments (10). The reduction in these characters is reflected in the number of ears produced, the length of the ears, the number of spikelets and grains per ear and, ultimately, in the grain yield.

7. Even after the elimination of the effect of these characters determining yield, the correlation between the yield per ear and the mean maximum temperature of the ripening period turned out to lack significance. This is ascribed to the sterility of the pollen grains which increases both under SD- and LD-treatments resulting in a reduction in the fertility ratio and consequently in the grain yield under these treatments.

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MINERAL NUTRITION OF THE COTTON PLANT[1]

FRANK M. EATON[2] AND DAVID R. ERGLE

Field Crops Research Branch, Agricultural Research Service, U. S. Department of Agriculture and The Texas Agricultural Experiment Station, College Station, Texas

The accumulations and partitions of mineral elements and dry matter in upland cotton plants were followed: A, at five-day intervals for thirty days starting with the seed with the plants growing in Hoagland's solution in a warm-to-hot summer greenhouse, and B, at 15-day intervals from thirty days after planting to maturity in field plantings.

The latter data are not original but are recalculated from Olson and Bledsoe (16) who sampled cotton growing on Cecil sandy loam in Georgia; their results were the averages of two fertilizer treatments for the years 1939 and 1940 on a soil that had a pH 5.7, 2% organic matter, and which produced 2,000 pounds per acre of seed cotton. The data were reported by them as pounds-per-acre increments of N, P_2O_5, K_2O, CaO MgO, and plant weight for 15-day periods, together with the end-of-season totals. The Olson-and-Bledsoe data long have been regarded by us as especially meritorious and deserving of the additional analyses and interpretations which we have undertaken in this paper.

As an orienting foreword, some rather marked differences occurred in the mineral accumulations, and particularly in growth rates between the plants grown in the greenhouse in Hoagland's solution for thirty days and those first sampled in the field by Olson and Bledsoe at a like age after development during the typically cool conditions of the spring. The kernels of the Empire seed planted in the greenhouse had an average dry weight of 0.063 gm; at 30 days the leaves and stems had dry weights of 2.93 gm. In the field at thirty days the plant tops weighed only 0.13 gm. The greenhouse-to-field ratios of mineral accumulations (percentages on dry weight of leaves and stems) at thirty days were N, 4.0 to 3.5; P, 0.8 to 0.3; K, 4.5 to 2.7; Ca, 3.3 to 3.1; and Mg, 0.7 to 1.4.

Methods

Olson and Bledsoe (16) used "Official Methods" for the determination of N, P, and Ca. Magnesium was determined by the 8-hydroxyquinoline method and K by the Wilcox (23) procedure. Our measurements were by "Official Methods" (Assoc. Agr. Chem., ed. 4) for N, P, Ca and Mg; K was determined as above and Na by the flame photometer.

The greenhouse plants were germinated for five days in washed river sand copiously watered with Hoagland's solution (plus micronutrients, sequestrene NaFe and 1 Mm/l of NaCl) and transferred on the fifth day to the same solution aerated in a series of twelve 20-liter stone jars. The first sample was of 100 seed kernels, the second at five days was of 60 plants after emergence on fourth day, and the last at 30 days was of 17 plants. On the latter date the plants had 7 to 8 true leaves. Olson and Bledsoe started at 30 days from planting with replicated 100-plant samples and ended at day 150 with samples of 5 to 10 plants.

As recalculated to percentage compositions and cumulative grams of the various mineral elements per plant, the Olson-and-Bledsoe data (16) were found to be remarkably consistent for field samplings. In the instance of plant weight and the potassium percentage at 90 days, however, there were marked irregularities. We have taken the liberty in these two instances of using new values obtained by interpolations.

Results

Growth and Mineral Accumulations on Hoagland's Solution: The data from the greenhouse experiment are reported: a) graphically in figure 1, as the percentages at successive dates of the six elements as found in the leaf blades, stems, roots, and in entire plants, and b) in table I, as the weights per entire plant of these elements.

During the first five days of the experiment there were sharp increases in the concentrations of N, K, Ca, and Na. These rapid accumulations took place even though during the first four days the seedlings had not emerged from the sand. There was no change in dry weight between seed kernel and the 5-day-old seedlings; such respiratory loss of dry matter as may have occurred during germination was evidently regained by photosynthesis during the fifth day. One rather unexpected feature of the data was the loss in weight per plant of P during the first five days and the continued decrease in P concentration during the first 15 days. The gain in Ca (extremely low in the seed) was continuous during the full 30-day period,

[1] Received July 19, 1956.

[2] Present address: Department of Soils and Plant Nutrition, Citrus Experiment Station, Riverside, California.

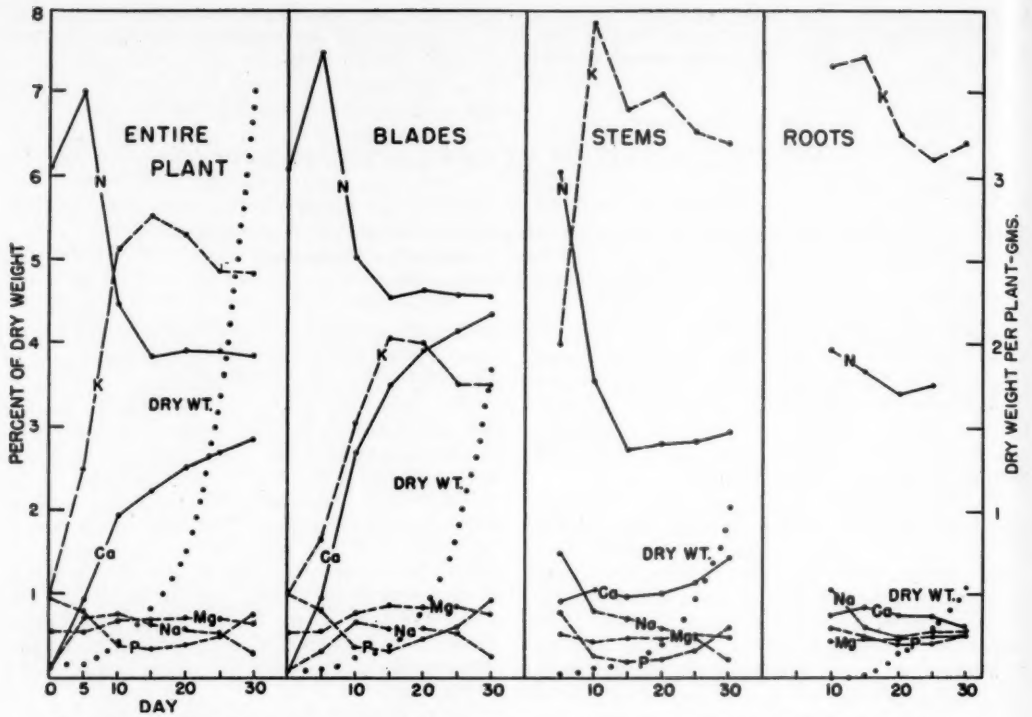

FIG. 1. Growth and percentage composition of cotton plants grown for 30 days in Hoagland's solution.

whereas Mg concentration remained almost constant. Nitrogen concentration increased only through the first five days and then decreased. Except for the loss of P and the continued increase in Ca, the first 15 days of growth (third true leaf one or two cm in width) accomplished the transition in mineral percentages from seed to seedling and balanced the proportions of the various ions near the levels that were to be maintained during the next 15 days of growth. Much has been said in the literature about mineral changes with ontogeny. At no later stage in the life cycle of the cotton plant are there transitions as rapid, or as radical, as those which occurred during this early cotyledonary stage of development.

The rapid uptake, during the first five days after planting, of Ca, along with N and K and the simultaneous loss from the plant of a part of the initial P, seemed rather surprising. Starting with only 0.08 mg/kernel of Ca, the seedling plants at 5 days contained 0.61 mg. The mechanisms for transporting Ca to the developing ovules appear to be deficient in the cotton plant. The hulls of the cotton seed contain only about the same amount of Ca as do the kernels. Presley and Leonard (18) after germinating cotton

TABLE I

DRY WEIGHT OF ENTIRE COTTON PLANTS AND WEIGHTS PER PLANT OF ELEMENTS DURING THIRTY DAYS IN HOAGLAND'S SOLUTION STARTING WITH DATA FOR THE SEED KERNELS. (PLANTS EMERGED ON DAY 4)

DAY	DRY MATTER	N		P	K	Ca	Mg	Na
		PROTEIN	TOTAL					
	gm	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*
0	0.063	3.68	3.83	0.60	0.63	0.08	0.34	0.04
5	0.066	2.38	4.61	0.52	1.64	0.61	0.36	0.47
10	1.97	5.42	8.75	0.73	10.1	3.80	1.30	1.52
15	3.68	7.29	14.1	1.18	20.4	8.16	2.50	2.31
20	7.54	19.0	29.4	2.86	39.6	19.0	4.98	4.30
25	16.7	42.8	65.0	8.19	81.0	45.0	11.9	9.21
30	34.8	89.5	135.9	26.5	168.8	99.8	21.9	9.80

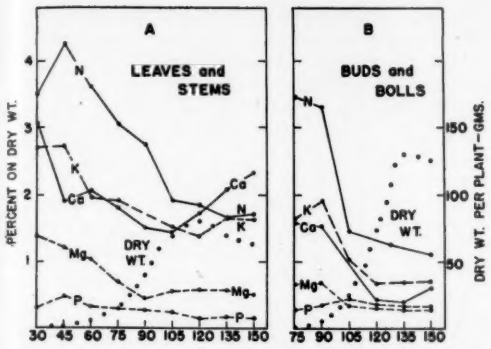

FIG. 2. Growth and percentage composition of cotton plants in the field on Cecil sandy loam soil between 30 and 150 days from planting.

seed on filter papers with water found the subsequent growth to be more dependent upon a supply of calcium than any of the 8 species compared. Mason and Maskell (14) were unable to find evidence that Ca is mobile in the phloem of cotton and emphasized the need for a continuous supply. Joham (13) observed that with Na present, the impact of transferring cotton plants from plus-Ca to minus-Ca was lessened somewhat.

The percentage compositions of the stems and roots attract attention largely by the fact that whereas N and K were especially high, P, Ca, Mg, and Na were all grouped near or below the 1 % level. At 30 days, Na was higher in the roots than in the stems or blades; otherwise there is little evidence of any marked Na localization such as has been reported in the literature for a number of other plants.

Starting on day 10, the weights/plant of dry matter and of each of the minerals (P and the last Na value excepted) tended to double with rather remarkable regularity during each 5-day interval.

FIELD PLANTINGS ON CECIL SANDY LOAM: The data by Olson and Bledsoe are presented in figure 2 as percentage compositions on dry weight of cotton plants between the ages of 30 and 150 days when grown on a relatively rich soil under better than average rainfall conditions. These authors noted that the rainfall during each of the two seasons averaged six inches above normal for the period June, July, and August. The stems + leaves and the buds + bolls were collected and analyzed separately. The involucres of buds and bolls are prominent in the cotton plant and resemble leaf tissues; in well fruited upland cotton plants, the ratio of involucral area to leaf area is in the order of 1 : 3. Early in the fruiting period, the involucres contribute substantially to the dry weight of bud + boll samples but with the enlargement of the early bolls, the weights of the involucres become progressively secondary.

Between 30 and 45 there were both losses and gains in the concentrations of the 5 elements (fig 2). Day 105 initiated the period of rapid gain in the weight of the reproductive tissues. But it was before, rather than afterwards, that large declines in the concentrations of N, P, K, Ca, and Mg occurred. These declines took place both in the vegetative and reproductive tissues; only P in the buds and bolls gained a little before day 105. From day 105 onward the changes in the concentrations of all elements (except Ca which increased) were minor, with small declines being the rule. It was between days 105 and 150 that the buds and bolls came to constitute 65 % of the total dry weight of the entire plant (figs 3 and 4).

The relative position of N, P, K, and Mg were much the same in the vegetative and reproductive tissues but the concentrations were rather uniformly lower in the latter.

The 2,000 lbs/acre of seed cotton obtained during the two years of the experiment on Cecil sandy loam was on the basis of 23,000 plants per acre. The data are recalculated in figure 3 to show the grams/plant of dry weight (divided by 100) and of the five elements. The noteworthy point in these data is that after day 105 there was little change in either the dry weights of the leaves + stems or in the grams/plant of the five elements in these tissues. There was, however, a moderate loss of N from the leaves and stems between days 120 and 135. The tendency toward a

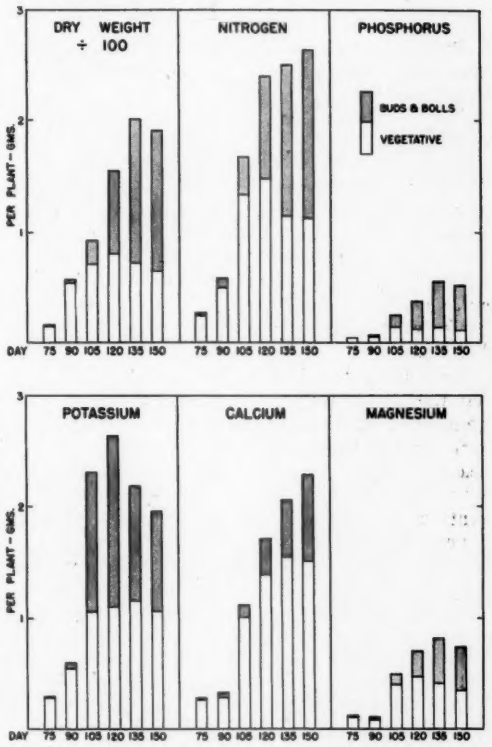

FIG. 3. Dry wt and nutrients (gm/plant) in vegetative and reproductive tissues.

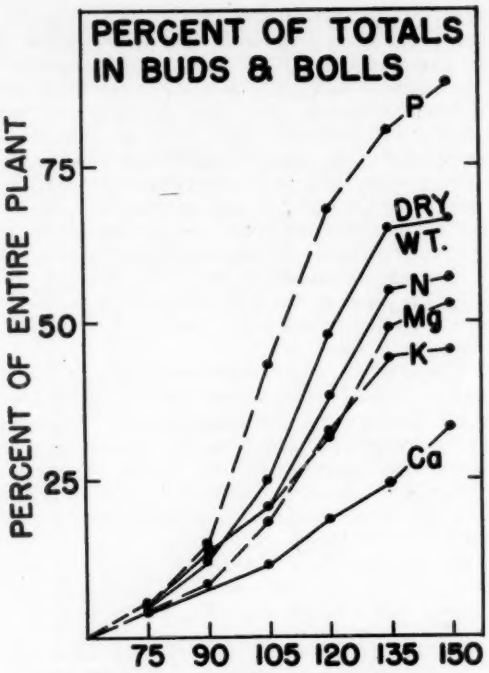

FIG. 4. Percentages of total dry matter and minerals found in the reproductive tissues from the start of reproduction until day 150.

leveling off in weight of Ca in the leaves and stems was delayed some 15 days longer than that of the other elements. Previous data have shown (8) that over the main part of the boll-setting period, the carbohydrate levels in the stems of irrigated cotton also tend to remain remarkably constant.

After day 105 the gains in the dry weight of the entire plants (tops) and the increments in mineral uptake were represented almost entirely by materials that were transported through the vegetative tissues to the squares and bolls. This is so nearly the case that the percentages of the total dry weight and of the totals of the individual elements found in the bolls at the end of the season, figure 4, provide a general measure (other than for Ca) of the total increments in dry weight and minerals that occurred after the first month of boll setting. In other words, the vegetative tissues became almost completely subservient to fruiting activities, but in becoming subservient they neither lost nor gained appreciably in weight or composition.

When some allowance is made for the loss of old leaves by abscission, for the loss of K from the buds and bolls, and for the continued gain in weight/plant of Ca, the growth and the mineral-uptake graphs are sigmoid flexures with the mid-points lying between days 90 and 105, at which time fruiting activities began to dominate the growth behavior.

Cotton is evidently at variance with highly determinant plants in the maintenance of its mineral compositions in the vegetative tissues during fruiting. Miller's voluminous data (15) on wheat show that the concentrations of N, P, and sugars found in the leaves and stems were only 25 %, or less, as high during late heading as they had been during the early boot stage. The concentrations of K in the leaves and stems of wheat were also down during heading but not as far as the constituents just mentioned. Ontogeny, except in the seedling stage, has little evident significance in the mineral accumulations of cotton beyond the fact that the rates of accumulation were slowed during the boll period in the same general proportion as the gain in total weight was slowed. But with fruiting, the final disposition of accumulating materials shifts from the vegetative to reproductive tissues.

The relatively large loss of K from the cotton bolls during the last 30 days appears to be a somewhat magnified instance of a much discussed subject. Miller (15) found examples of a net late-season loss of K from all tissues of wheat but such loss occurred in the instance of only two out of his 6 plantings; there was a loss from the heads in a third planting.

Olson and Bledsoe, as well as White (21), obtained data on the composition of entire cotton plants growing on other soils. The following data from Olson-Bledsoe (16) illustrate the limited influence of soil fertility on the mineral compositions of cotton. At the early-square stage the percentage compositions of entire plants were as follows.

SOIL	FINAL YIELD	N	P_2O_5	K_2O	CaO	MgO
	Lbs/acre			*% Composition*		
Tifton sandy loam	495	3.6	0.70	3.4	3.4	1.0
Clarksville gravelly loam	807	3.2	0.60	2.7	3.1	0.7
Cecil sandy loam	2000	3.4	0.63	3.0	2.8	1.1

Data such as these are in harmony with the relatively small differences occasionally found by others in the mineral compositions of plants grown on diverse soils. Duncan (5), who reported on corn and soybean grain and on brome and timothy hay growing on fertilized and on a badly depleted soil of the same type in Michigan found the differences in compositions of the crops between seasons to be greater than the differences between the two soils in any one year. Sims and Volk (20), on the other hand, report wide variations in the mineral compositions of given vegetables when grown on different soils in Florida.

COTTON AND OTHER PLANTS WITH RESPECT TO EARLY AND LATE ACCUMULATIONS OF MINERALS AND DRY MATTER: The data of table II were compiled as a means of comparing cotton with other plants in terms of the ratios (percentages) of early to late growth in the accumulations of dry matter and of minerals. The eight crop plants, four dicots and four monocots, are among those for which analysis had been made at an early stage when the young plants

TABLE II

PERCENTAGES OF LATE-GROWTH DRY MATTER VERSUS LATE-GROWTH MINERALS FOUND IN YOUNG PLANTS. (VALUES FOR MINERALS HIGHER THAN FOR DRY MATTER INDICATE THE EXTENT OF EXTRA MINERAL-UPTAKE DURING EARLY GROWTH)

PLANT	DRY MATTER	N	P	K	Ca	Mg	S
			Dicots				
Cotton *	2.8	6.4	3.1	5.1	5.5	7.1	...
Tobacco **	2.5	5.8	3.8	3.5	2.4	2.9	2.9
Tomato †	2.1	3.5	3.1	2.8	3.1	4.7	...
Potato ††	3.3	5.0	6.5	4.2	3.3	4.1	...
			Monocots				
Barley ‡	2.1	12.1	5.2	10.3	5.6	5.4	...
Winter wheat ‡‡	2.3	7.8	6.7	6.6	...	...	...
Corn [a]	2.0	4.7	2.5	9.0	...	...	...
Sugar cane [b]	1.4	10.0	3.1	5.1	4.6	3.7	...

* Olsen and Bledsoe (16), 60 vs 135 days; the latter included the bolls which constituted 65 % of the total.

** Grizzard, Davies, and Kangas (11), 21 days vs 63 days after transplanting; leaves and stems only.

† Hester (12), one month after transplanting vs two months later; the latter included fruits which constituted 41 % of the total.

†† Carolus (4), approx. values taken from graphs (pounds/acre) 42 vs 91 days after emergence; the latter included the tubers which constituted 42 % of the total.

‡ Burd (3, table 2), 5 weeks after planting vs 3 wks before ripening; the latter included the heads which constituted 41 % of the total.

‡‡ Miller (15, 1933–34 season, means of two varieties), Dec. 13 vs June 6, the latter included chaff and grain (early dough stage) which constituted 30 % of the total weight.

[a] Sayre (19), 30 vs 91 days after planting; the latter (Sept. 3) included the grain which constituted 35 % of the total.

[b] Ayres (2), 2 vs 14 months; leaves and stems only.

weighed in the order of 2 or 3 % of the weight reported at some late date. As may be observed in the footnotes, the 2 to 3 % stage of development was customarily attained during the second month of growth. Substantial losses of K, and occasionally of other minerals, as well as of leaves, are sometimes noted during advanced growth; to avoid such aberrations, data several weeks in advance of final harvest are used for barley, wheat and corn. The next to final harvest was used for cotton and the tobacco, tomato, and potato plants were not mature. Of the 8 crops, six had a substantial part of the final weight in reproductive tissues; only tobacco and sugar cane were composed entirely, or nearly so, of stem and leaf tissues. In no instance, except for potato, are underground parts included.

If cotton occupies a distinctive position with respect to its early accumulations of minerals, it would appear to lie in its disproportionate uptake of Ca and Mg during early relative to late growth. And, conversely, in the restricted early uptake of P. The high relative uptakes of Ca and Mg by cotton during the first two months of growth are shown by the values 5.5 and 7.1 which are to be compared with the 2.8 value for dry matter. Stated otherwise, during the first two months of growth, this field cotton accumulated both Ca and Mg at rates double or more the gain in dry matter. The P ratio for cotton is 3.1 whereas that for wheat is 6.7; wheat had an early-to-late dry-matter ratio of 2.3. Corn as studied by Sayre had a phosphorus value that was not out-of-line with cotton: 2.5 for P and 2.0 for dry matter. Sayre's value for corn seemed a little surprising since in studying other literature on the P accumulation by cereals (10, 22), the writers had developed the notion that the cereals accumulated large reserves of phosphorus while young. Rather than being characterized by rapid early accumulations of phosphorus, it appears, instead, that cereals are noteworthy primarily in the extent to which they transfer some of their minerals, as well as sugars, out of the leaves and stems into the heads during late growth. Petri (17) and Williams (22) have emphasized the early accumulations of nitrogen in certain cereals and its later massive movement into the reproductive tissues. Table II indicates that barley, wheat and cane are more avid early accumulators of nitrogen than either cotton or corn.

Even though there is little or no exhaustion in the vegetative tissues of cotton as a result of the transport of minerals or sugars to the bolls during fruiting, a marked exhaustion of P in the vegetative tissues of cotton does take place when the supply is removed (9). Arnon et al (1) have shown that the fruit of the tomato plant has a high P-priority during P-starvation.

DISCUSSION

The explanation for the uniformity in the vegetative structures of the cotton plant as regards their weight and their minerals during the period of heavy boll development must lie in the fact that cotton has the remarkable characteristic of shedding young squares and bolls that cannot be brought to normal maturity. No instance has been recorded of an over-fruiting of the cotton plant that resulted in a significant reduction in weight/boll—providing, only, that conditions remained reasonably suitable for growth and boll development. Although over-fruiting, with consequent poor fruit weights, is common among horticultural plants generally, in cotton, within a variety and climatic complex, the number of bolls developed tends to remain proportional to the weight of the leaves and stems.

The actual nature of the chemical stimuli responsible for the drying up of small floral buds and for the abscission of young bolls remains unknown but it is believed (7, 8) that developing cotton bolls secrete a mobile growth-inhibitive substance of anti-auxin nature which causes enough of the bolls to abscise to maintain a balance between fruiting activities and the vegetative stature of the plant. This boll-setting inhibitor is evidently responsible, also, for the suppression of vegetative growth after boll setting has progressed; when the earliest bolls set by well-fruited

cotton plants start to open, it is usual for the plant to renew its vegetative growth and at the same time to start to set new bolls. Because of the maintenance of carbohydrate and nitrogen in the vegetative tissue and, as shown in this paper, of the minerals in general, the old nutritional theory of boll shedding is regarded as inadequate.

The rate of mineral accumulations by the cotton plant are shown in figure 3 to be maintained well during fruiting. The maintenance of mineral uptake is undoubtedly related to the simultaneous maintenance of sugars at levels ample to support the respiratory activities involved in mineral accumulations. Conversely, the background activities that support photosynthesis are dependent upon the maintenance of mineral concentrations in the leaves.

Considered in the light of the foregoing, the cotton plant, through the agency of boll shedding, has developed a rather remarkable mechanism for the maintenance of the mineral and carbohydrate levels essential for the continued production of high quality fibers throughout the period of boll development. New bolls are customarily set by the cotton plant over a period of 4 to 5 weeks in July and early August. Each boll in upland cottons requires 45 or more days before it opens. With any substantial deterioration in the nutrients of the vegetative tissues, deteriorations would result also in the length, strength, and weight/inch of the fibers. With marked deteriorations in any of these fiber properties, the cotton would become unsuitable for the spinning of high quality yarns.

The Olson-and-Bledsoe plants were heavily fruited with the weight of the bolls at 150 days constituting 66 % of the dry weight of the entire plants. After day 120 there was no gain in the dry weight of the leaves and stems. The data are regarded as representing the normal behavior of productive strains of upland cotton under suitable environmental conditions. Plant behaviors somewhat different from the foregoing can probably be expected here and there in the instance of cotton strains under growth conditions that cause them to be highly determinate and in the instance of plants which either by inheritance or because of insect damage are poorly fruited. In highly determinate cottons it is not unusual for the plants to lose color and appear senescent by the time the first bolls are about ready to open; something similar happens when the supply of N runs out after the plants have set bolls. In such plants it would not be unexpected to find late-season reductions both in the minerals and carbohydrates as fruiting progressed. Poorly fruited plants continue a vigorous vegetative expansion throughout the summer. In such plants one might expect to find mineral concentrations that correspond to the higher values observed in figure 2 in advance of fruiting. Increased concentrations of sugars and a stimulated uptake of certain minerals have been found (6) after bolls are removed from cotton plants. In well illuminated cotton plants it is usual to find 6 or 7 bolls/100 gm fresh stems and leaves but under muslin shades the number of bolls has been reduced to half. Large plants, with much self-shading, have lower relative-fruitfulness values than small plants.

Summary

1. The growth and mineral accumulations of cotton plants were measured at five-day intervals for 30 days. The plants were germinated and grown in Hoagland's solution in a warm-to-hot summer greenhouse. Between the seed kernels and five-day-old seedlings (emergence on day four) there was no change in dry weight nor in weight of Mg per plant but there were large accumulations of N, K, Ca, and Na, and a loss of P. At no later stage in the life cycle of the cotton plant are there such rapid or pronounced changes in mineral compositions. The concentration of P (not the wt/plant) declined to day 15 and then started to rise. The concentration changes between days 15 and 30 were for the most part minor. Between days 10 and 30 the weight/plant and the weights of the several elements showed strong tendencies to double at 5-day intervals.

2. As grown in Georgia on a fertile field soil with better than average rainfall (original data by Olson and Bledsoe) entire cotton plants showed sigmoid gains in dry weight and in mineral accumulations over a 150-day period; the time of the most rapid increases centered at, or a little before, 105 days. N and Ca increased to day 150 and the weight of K per plant decreased sharply after day 120.

3. During the development of the bolls, there were large increments in dry matter and in weight/plant of N, P, K, Ca, and Mg. Except for an extra retention of Ca in the vegetative tissues, these increments passed through the vegetative tissues to be deposited in, and to promote the growth of, the buds and bolls. During the heavy boll setting and the growth of the older bolls, there were neither substantial gains nor losses in the dry matter or minerals of the vegetative tissues.

4. At maturity the weight of buds and bolls constituted 66 % of the weight of the entire plant. The buds and bolls contained 57 % of the total N, 79 % of the P, 46 % of the then remaining K, 53 % of the Mg, but only 34 % of the Ca. As fruiting advanced there was a small net export of P from the vegetative to the reproductive tissue but between days 105 and 150 the weight of P/plant doubled.

5. The maintenance of uniform concentrations of minerals in the leaves and stems (and of carbohydrates in the stems) during heavy fruiting appears to characterize the reproductive cycle of the cotton plant. It is this characteristic which permits the cotton plant to produce uniform fibers over a prolonged period of boll development. The basis of this unique adaptive mechanism lies in the fact that through the agency of abscission the cotton plant discards superfluous bolls that could not be brought to normal maturity. The shedding of superfluous bolls is evidently induced by the secretion from developing bolls of an anti-auxin type of material which diffuses out through the plant

to curtail vegetative expansion and induce the abscission of new bolls as relative fruitfulness comes to exceed around 6 to 7 bolls/100 gm of fresh leaves and stems. Without shedding, the late-season fibers would inevitably be shorter, weaker, and finer and, thereby, poorly suited to the spinning of high quality yarns.

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A RE-EXAMINATION OF THE SUPPOSED EFFECT OF RIBOFLAVIN ON GROWTH[1]

C. L. MER

Research Institute of Plant Physiology, Imperial College of Science & Technology, London, S.W. 7, England

In 1949 Galston (9) published his observations on the photoinactivation in vitro of indoleacetic acid (IAA) by riboflavin (RFN) and this result was subsequently used by Galston and Baker (11) to explain the different behaviour of etiolated pea stem segments in light and darkness when grown in media containing IAA and RFN. It was reported that the elongation of these segments was promoted in darkness but inhibited in the light (11, fig 1, and table 1, p. 774). They considered, but found no evidence for, the suggestion that the observed behaviour of the pea segments was due to photodecomposition products of riboflavin and concluded that ". . . riboflavin exerts its inhibitory effect on growth by sensitizing the photoinactivation of auxin or some other growth factor. . . ." They found that native auxin from coleoptiles, collected in agar blocks by diffusion, was inactivated by added riboflavin in the light, and also that IAA was inactivated by a concentrated pea brei. Examining the matter further they showed that the action spectrum for phototropism was similar to that for the inactivation of IAA by riboflavin in vitro. While they

[1] Received August 1, 1956.

appreciated that the spectrum for phototropism was similar to the absorption spectrum for carotene, the accumulated evidence was considered sufficient to warrant the theory that riboflavin is a major photoreceptive agent causing the inactivation of auxin in both light-inhibited extension growth and phototropic phenomena.

Although light-inhibition of elongation and phototropism have both been referred to changes in auxin metabolism they seem in some respects to be different phenomena, e.g., extension growth of stems is most effectively reduced by red light (24, 32, 33) but is affected by blue light only slightly (32) whereas phototropic responses are most readily evoked by blue light and hardly at all by red light (10). Again phototropic perception does not take place under anaerobic conditions (1) but under these conditions illumination reduces the growth of stem tissues (23). This diverse behaviour makes an explanation in terms of a single reaction—the inactivation of auxin by riboflavin in the light—somewhat improbable.

With a view to ultimate experiments on the phototropic responses of coleoptiles with increased riboflavin content, both the entry of riboflavin into the plumule through the basal cut surface and its effect on the growth of the coleoptile were studied.

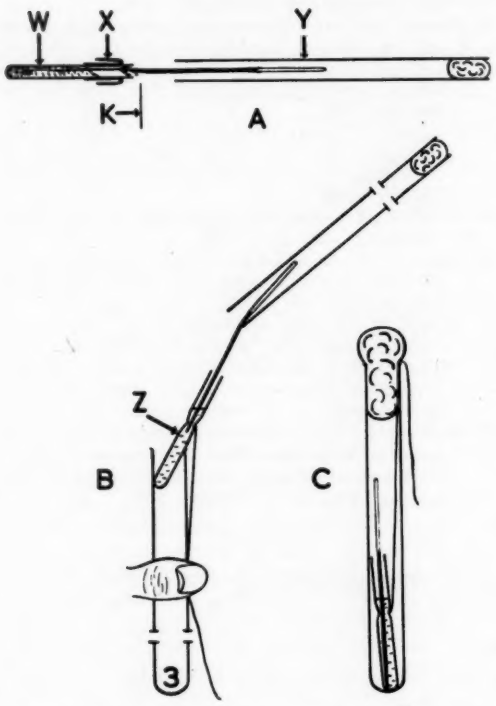

FIG. 1. The method of growing and treating the plants. A shows the upper tube (Y), lower tube (W) and connecting tube of silicone rubber (X). The plant was cut off just above the seed by pulling the upper and lower tubes apart and drawing the plant across the knife blade (K). The outlined upper part of the seedling is the coleoptile, the lower solid part is the mesocotyl. B shows the numbered test-tube with raised inner tube (Z) containing the test solution into which the severed plumule is being introduced. The constriction and the thread attached are also shown. C shows the severed plumule in position. The test-tube number has been omitted for clarity.

THE GROWTH OF SEVERED COLEOPTILES

MATERIALS AND METHODS: *Avena sativa* L. (Svalof Victory) was used in the majority of the experiments; in the remainder *Triticum sativum* L. (var. Pedigree) was used. The seed was supplied by Messrs. S. Finney & Sons of Newcastle.

The oats were dehusked and planted individually in glass cylinders (fig 1 A). The lower part (w) contained two short slips of filter-paper and was partially filled with culture solution (KH_2PO_4, 0.3 gm; $Ca(NO_3)_2$, 0.94 gm; $MgSO_4$, 0.49 gm; KNO_3, 0.3 gm; distilled water 1000 ml). The upper tube (y) was attached by a short length of silicone-rubber tubing (x). Before use the cylinders were sterilized; then opened under a sterile hood to plant a spergon-dusted seed between the filter papers; the upper tube was then replaced. After four days growth at 24° C in a thermostat, the length of the coleoptile was measured through the glass and the upper tube was pulled off so that the mesocotyl could be cut just above the seed by pulling the plant against a sterile knife blade (k) mounted on a stand (fig 1 A). The plumule, which remained in the upper glass tube, was transferred aseptically to a numbered test-tube containing the test solution in the inner tube (z, fig 1, B and C). A thread tied at the constriction passed out of the test-tube so that removal of the plug pulled up the inner tube and the plumule could be fed into it. The inner tube and plumule were then slowly lowered into the test-tube, which was replugged. If the plumule touched the inside of the test-tube it adhered to the glass and the inner tube descended without the plant; with practice, however, this was avoided. Thus the coleoptile itself was not immersed in the solution, which entered the plant via the transpiration stream.

The transferences and initial measurement of the coleoptile in each numbered tube were carried out in dim orange light, and after remaining for two hours in darkness those plants to be treated were illuminated for one hour (100 ft-c). All plants were then returned to the thermostat and after a further 3-days growth the coleoptiles were remeasured and the increments of growth obtained.

EXPERIMENTAL RESULTS, *Factorial Experiments ± IAA ± Riboflavin ± Light*: In these experiments four test solutions were employed which were sterilized before use: control (0.01 M phosphate buffer, pH 6.4 with 2 % sucrose) and control with IAA and riboflavin separately and together at a concentration of 1 ppm. These were duplicated for ± light in each of four blocks. There were 7 replicates per treatment

per block, making a total of 224 replicates for each experiment.

The initial lengths of the coleoptiles and the increments of growth for one experiment are shown in table I.

An analysis of covariance was carried out on these data using the method described by Snedecor (26, section 12.7). This analysis, referring to a nutritional experiment, is not carried to completion; the sum of squares attributable to each of the nutrients under discussion and their interactions are not individually computed. In the present instance, however, it was essential to estimate separately the main effects and their interactions and these are also recorded in table I. Variability due to blocks and their interactions which are not of immediate concern have been omitted.

The results show a highly significant effect of illumination, and a significant interaction between IAA and RFN, but no main effect of either IAA or RFN. The very large interactions anticipated between RFN and light and also between all three factors were absent and so that found between IAA and RFN appeared dubious, especially when the responses of the illuminated and dark sets were analysed separately. In the light the interaction IAA × RFN was not significant (F = 0.99) while in darkness it was highly so (F = 4.74; 3.94 is required for significance at 5 % level). In two further experiments this interaction was negligible (F = 0.53 and 0.04) while in a third it approached significance (F = 3.58). IAA and RFN do not interact in vitro in darkness and they therefore would not be expected to do so in vivo.

The concentration of IAA and RFN (1 ppm) used in these experiments was that found to be effective by Galston and Baker (11) for pea epicotyl segments. In the absence of similar effects in these experiments further work was undertaken with IAA and riboflavin separately to find effective concentrations which could later be used in a composite experiment.

Experiments with IAA Alone: A range of concentrations from 100 ppm to 0.1 ppm were used but significant effects were only found with the higher concentrations. The result of an experiment employing 100, 50, 25, 0 ppm is recorded in table II together with the analysis of covariance.

The effect of increasing the concentration of IAA is apparently to decrease the growth of the coleoptile. Quite apart from the variation in initial length the increments show a step-wise decrease as the concentra-

TABLE I

EFFECT OF IAA, RFN AND LIGHT ON THE GROWTH OF SEVERED OAT COLEOPTILES

BLOCK NO.	IAA + RFN		IAA		RFN		CONTROL	
	X	Y	X	Y	X	Y	X	Y
Not illuminated								
1	91	148	119	156	124	162	122	159
2	103	148	109	152	92	157	109	142
3	93	160	91	163	97	161	89	147
4	93	152	102	157	89	170	100	154
Total	380	608	421	628	402	650	420	602
Illuminated								
1	117	98	107	105	94	115	101	104
2	90	115	107	103	91	116	100	101
3	95	124	97	118	110	100	97	108
4	103	104	103	117	95	120	100	115
Total	405	441	414	443	390	451	398	428

X = Total initial length in mm. Y = Total increment of growth in mm.

Measurements of 7 replicate coleoptiles per treatment per block ± 1 ppm indoleacetic acid (IAA) and riboflavin (RFN) as indicated in 0.01 M phosphate buffer with 2 % sucrose at pH 6.4. Pretreatment of 2 hrs in these solutions in darkness, then ± illumination (100 ft-c for 1 hr). Plants grown 3 days after treatment.

ANALYSIS OF COVARIANCE

	D.F.	MEAN SQ.
RFN	1	6.10
IAA	1	0.40
Light	1	2631.68 ††
IAA × RFN	1	41.74 †
RFN × light	1	0.01
IAA × light	1	3.77
IAA × RFN × light	1	9.61
Error	191	7.91

† Significance, 5 %.
†† Significance, 1 %.

TABLE II

EFFECT OF IAA ON THE GROWTH OF SEVERED OAT COLEOPTILES

IAA, PPM	MEASUREMENTS *	NOT ILLUMINATED	ILLUMINATED
100	X	538	542
100	Y	529	351
50	X	531	497
50	Y	592	382
25	X	552	475
25	Y	650	418
0	X	592	502
0	Y	715	446

* X = Total initial length in mm. Y = Total increment of growth in mm.

Measurements of 28 replicate coleoptiles; 2 hrs pretreatment in darkness then ± light (100 ft-c) for 1 hr. Solutions: phosphate buffer (0.01 M with 2 % sucrose, pH 6.8) + IAA as indicated. Final measurements 3 days after illumination.

ANALYSIS OF COVARIANCE

	D.F.	MEAN SQ.
IAA	3	266.61 ††
Light	1	3711.30 ††
IAA + light	3	38.14 †
Error	191	8.92

† Significance, 5 %.
†† Significance, 1 %.

tion increases. This result agrees with that recorded by Avery and La Rue (2), who similarly found a reduction in growth when IAA was applied to cut-off coleoptiles through the exposed basal surface.

Experiments with Riboflavin Alone: Experiments carried out with concentrations of riboflavin up to 60 ppm gave entirely negative results.

In all these experiments the increment of growth was obtained three days after the beginning of treatment, but in the following experiment daily increments of growth were observed in case a transient effect of riboflavin was escaping observation. The result is recorded in table III.

These data again show neither an effect of riboflavin nor an interaction with light.

Entry of Riboflavin into the Plumule: In view of the failure to detect an unequivocal effect of riboflavin on the growth of the coleoptile its entry into the plumule was directly determined. Plants were cut off as described and dipped into a solution containing 60 ppm of riboflavin (in phosphate buffer pH 6.8 with 2 % sucrose). After treatment for 2 or 24 hours in darkness they were dissected into three parts

TABLE III

DAILY GROWTH OF SEVERED OAT COLEOPTILES WHEN SUPPLIED WITH RIBOFLAVIN

DAYS AFTER END OF ILLUMINATION	MEASUREMENTS *	ILLUMINATED		NOT ILLUMINATED	
		+RFN	-RFN	+RFN	-RFN
1	X	133	122	133	126
	Y	65	64	76	83
2	X	119	151	135	146
	Y	125	116	162	163
3	X	112	129	121	134
	Y	157	150	224	217
4	X	159	156	144	140
	Y	123	127	225	224
5	X	123	133	117	144
	Y	135	156	264	239
6	X	136	141	125	123
	Y	148	149	245	269

* X = Total initial length in mm. Y = Total increment of growth in mm.

Measurements of 8 replicates per treatment per day. Solution: 0.01 M phosphate buffer, pH 6.7 ± sucrose, 2 %, and riboflavin, 60 ppm. Pretreatment 2 hrs in darkness, then ± light (100 ft-c) for 1 hr, then darkness for remainder of growing period. First measurement 24 hrs after termination of illumination.

ANALYSIS OF COVARIANCE

	D.F.	MEAN SQ.
RFN	1	18.34
Light	1	3723.27 †
RFN × light	1	0.01
Error	185	10.89

† Significance, 1 %.

TABLE IV

ESTIMATION OF THE RIBOFLAVIN (RFN) CONTENT OF OAT PLUMULES DIPPED IN SOLUTION A CONTAINING 0.01 M PHOSPHATE BUFFER WITH 2 % SUCROSE ± 60 PPM RFN

PLUMULE PART	RFN CONTENT, μGM/GM DRY WT			
	2 HRS		24 HRS	
	-RFN	+RFN	-RFN	+RFN
	A. pH 6.8			
Coleoptiles	35.9	25.9	22.9	88.4
	37.2	27.5	22.8	51.2
Mesocotyl (upper half)	27.2	21.0	20.0	72.8
	18.0	18.5	23.3	66.4
Mesocotyl (lower half)	27.6	129.0	17.6	432.0
	28.2	125.0	22.3	581.0
	B. pH 9.5			
Coleoptiles	33.0	33.6	30.5	125.6
Mesocotyl (upper half)	18.1	19.6	23.8	....
Mesocotyl (lower half)	29.1	102.4	33.3	465.9

—coleoptiles and upper and lower halves of the mesocotyl. The lower half of the mesocotyl which had been immersed in the solution was thoroughly rinsed to remove any superficial riboflavin. The material was dried, weighed and assayed for riboflavin content using the standard microbiological technique employing *Lactobacillus casei* (21). The results in micrograms of riboflavin per gm dry weight for two experiments in each treatment are recorded in table IV *A*.

These results show that in a 2-hour application riboflavin did not reach the coleoptile but remained in that part of the plumule actually immersed in the solution. In 24 hours, however, the content of all parts of the seedling had increased.

The failure therefore to detect the effects of riboflavin on growth was due to the use of a 2-hour pretreatment period, for in this time riboflavin did not reach the coleoptile. This slow movement was in marked contrast to the behaviour of eosin, which reached the tip of the coleoptile in 2 to 3 minutes. It was erroneously assumed that riboflavin similarly applied would travel as fast and that 2 hours would therefore provide ample time for it to pervade the tissues.

In 24 hours, however, the riboflavin content of the coleoptiles had increased considerably. It might be expected therefore that its prolonged application in darkness would lead to a promotion of growth as recorded by Galston and Baker (11). In this connection some of the results already mentioned may be reconsidered, particularly those referring to the plants treated with riboflavin in darkness. Although these plants were supplied continuously with riboflavin during the 3-day growing period, they showed no promotion of growth. Also, the plants whose measurements are recorded in table III were treated for 1 to 6 days, but these, too, showed no significant growth promotion. Nor did they display a depression which

usually follows the administration of metabolically active substances in grossly supraoptimal concentrations. It would appear therefore that riboflavin does not readily enter the protoplast.

The very large effect of light which appeared in all these experiments requires further consideration. From the foregoing statements its depressant effect on growth cannot be due to variation in riboflavin content and it must consequently be due to some other factor. All the accumulated data were therefore re-examined, in particular the relation between the increment of growth and the initial length of the coleoptile. The 224 individual observations of experiment 1 (table I) separated into light and dark sets are shown plotted in figure 2. It is clear that the initial length of the coleoptile is an important factor in determining the growth increment after a short exposure to illumination. Moreover, in darkness there is a positive correlation between the measurements (slope = 0.178) while after illumination the correlation is strongly negative (−0.539). As the slopes of these regressions are significantly different ($t = 7.8$) a strict covariance analysis is illegitimate.

This discrepancy between illuminated and dark plants is a matter of some importance in connection with the use of tissue segments for such experiments. By cutting sections of uniform length from a variable plant population the effect of the initial length of the plant on the subsequent growth of the segments cannot be determined and no correction can be made for differential responses in light and darkness.

An explanation of this different behaviour may be tentatively made on the lines suggested by Thompson (29, 30) namely that the longer coleoptiles are more mature, and as the effect of light is to hasten maturity they consequently elongate less than shorter coleoptiles which are less mature.

Alternative Methods of Raising the Riboflavin Content of Coleoptiles: 1) Charles (5) has directed attention to the slow movement of basic dyes and of some antibiotics into leaves whose cut petioles were immersed in solutions of the substances. He attempted to explain the phenomenon by suggesting that the xylem walls were negatively charged so that positively charged molecules would tend to be retained. Similar results have been reported by Crowdy and Pramer (6, 7) and Pramer (25).

With a view to facilitating the movement of riboflavin in oat plumules an experiment was carried out with a riboflavin solution buffered to pH 9.5.

The plants were grown as previously described and the severed plumules were dipped into a solution of riboflavin (60 ppm) for 2 or 24 hours in darkness. Some plants were then assayed for flavin content while others were exposed to light (100 ft-c) for one hour, yet others remaining in darkness as controls. While exposed the coleoptiles of the plants were measured to determine their growth during pretreatment

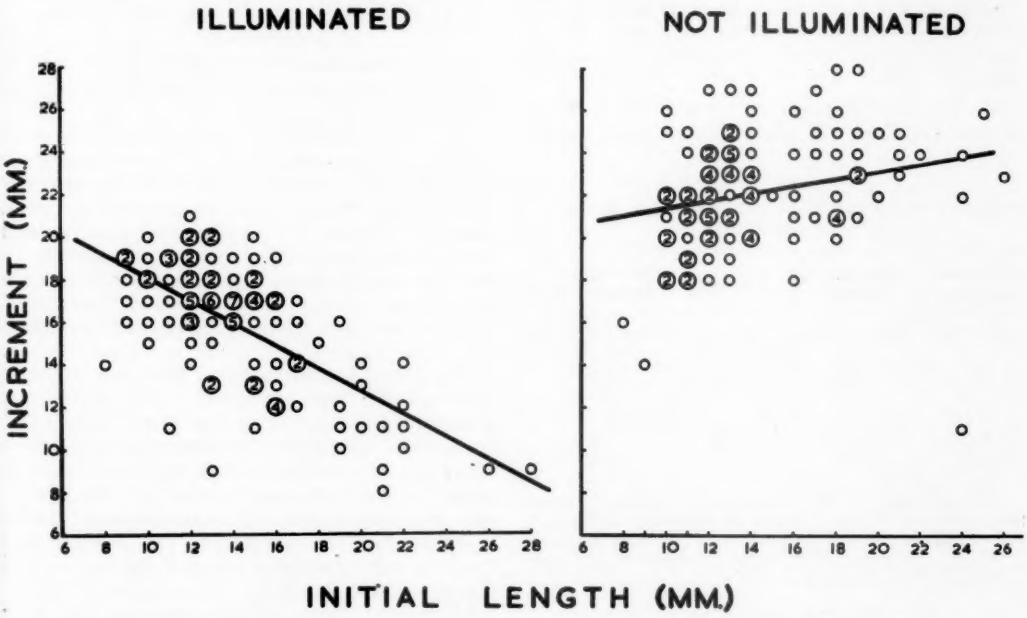

FIG. 2. The relationship between the initial length of the coleoptile and increment of growth in 3 days in darkness and after a 1-hr exposure to light (data of table I). Individual observations (112) in each set (± light) comprising the four treatments (IAA + RFN; IAA alone; RFN alone and controls), which are not separately indicated. Single circles are single observations. The numbers in the circles refer to the number of observations occurring on the same point. Regression slopes: Illuminated = −0.539; not-illuminated = 0.178.

and the corresponding information for the dark controls was obtained by exposing these plants very briefly to dim red light and measuring them. After treatment the plants were remeasured after a further 48 hours growth in darkness at 24° C.

The results of the flavin analyses are entered in table IV *B*. When these are compared with those recorded in table IV *A* (solution at pH 6.8) it will be seen that the results are very similar in that no riboflavin reached the coleoptile after a 2-hour treatment but it had done so in 24 hours. These latter plants were thus known to have a high content of riboflavin and they were therefore suitable for testing interaction between riboflavin and light on the growth of the coleoptile. The initial measurements and increments of the plants pretreated for 24 hours are shown in table V. The data appertaining to the 2-hour pretreatment are not quoted as no effect on growth was found.

Because of the inapplicability of a strict covariance analysis already mentioned the data for the illuminated and dark sets of plants were analysed separately, and the interaction between riboflavin and light was evaluated by a *t* test.

The effect of illumination is immediately apparent from the figures; it will also be seen that there was no effect of riboflavin on growth nor an interaction with light. The absence of an interaction could not have been due to lack of riboflavin in the coleoptile, it must be attributed either to the failure of riboflavin to penetrate the protoplast or, if entry did occur, to the absence of a reaction with the naturally occurring auxin.

2) Oat and wheat plants were grown in a culture medium containing 60 ppm of riboflavin for 5 days.

TABLE V

EFFECT OF RIBOFLAVIN ON THE GROWTH OF SEVERED OAT COLEOPTILES

INITIAL LENGTH, MM	PRE-ILLUM. INCREMENT, MM	LIGHT, FT-C	POST-ILLUM. INCREMENT, MM
	−RFN		
444	310	100	99
483	278	0	231
	+RFN		
477	316	100	93
482	276	0	236

Measurements of 26 replicates per treatment. Riboflavin solution (60 ppm) in 0.01 M phosphate buffer with 2 % sucrose at pH 9.5. Pretreatment 24 hrs in darkness, then light for 1 hr. Final measurements 48 hrs after termination of illumination.

ANALYSIS OF COVARIANCE

Effect of riboflavin—a. Pretreatment increment: $F = 0.46$
b. Post-illum. increment:
Illuminated: $F = 0.54$
Not illum.: $F = 0.09$
RFN × light: $t = 0.53$

TABLE VI

FLAVIN CONTENT OF OAT AND WHEAT PLANTS GROWN IN RIBOFLAVIN SOLUTION (60 PPM) FOR FIVE DAYS

PLANT PARTS	FLAVIN CONTENT (μGM/GM DRY WT)	
	−RFN	+RFN
	Oat	
Coleoptiles	25.1 23.0	24.2 22.6
Mesocotyl (upper half)	15.7 14.2	14.1 13.7
Mesocotyl (lower half)	23.4 18.9	27.5 26.8
Roots	23.2 15.2	466.0 402.0
	Wheat	
Coleoptile (upper half)	8.9	10.0
Coleoptile (lower half)	9.8	9.8
Roots	14.7	300.3

They were then dissected and assayed for flavin content. The results are recorded in table VI.

These results show that riboflavin applied to the seedlings in this way failed to reach the coleoptiles; in all probability it failed to enter the plants at all, as the high value was most likely due to the adsorption of riboflavin on the external walls of the root system.

THE GROWTH OF WHEAT COLEOPTILE SEGMENTS

It has been shown that riboflavin moves slowly in the xylem of oat plumules and that it fails to influence the growth of the coleoptile.

If it be true as Charles (5) suggested that slowly moving molecules are bound by electric charges to the xylem walls, there is a high probability that once attached they remain so and do not enter the protoplast, even though as a result of immersion the tissue shows a high content of the particular molecule. It was thus important to repeat the previous experiments using segments of tissue actually immersed in the solutions, as was done by Galston and co-workers (11, 14).

MATERIALS AND METHODS: Wheat (var. Pedigree) was used for these experiments. The plants were grown for 4 days at 24° C, continuously swept with air (purified by passing it through an activated-carbon filter) saturated with water vapour. From each coleoptile a single 10-mm segment was cut after discarding a 3-mm tip section. The segments were prepared in dim red light and distributed 5 at a time into small specimen tubes containing 0.5 ml of the test solution. The tubes were then rotated on a klinostat (20) for a 3-hour pretreatment period in darkness, after which those to be illuminated were exposed for 0.5 to 1 hr to white light (400 to 500 ft-c). During exposure the segments were measured to provide information on their extension during pretreatment. After exposure the tubes were returned to the klinostat for a total

TABLE VII

EFFECT OF RIBOFLAVIN AND LIGHT ON THE GROWTH OF WHEAT COLEOPTILE SEGMENTS

LIGHT, FT-C	RFN CONC, μGM/ML				
	10	1	0.1	0.01	0
	Total increment, mm				
400	287.5	276.0	277.5	264.5	286.5
0	323.5	317.0	322.5	313.5	322.5
	Post-illumination increment, mm				
400	246.0	242.5	243.0	231.0	249.0
0	282.0	283.5	288.0	280.0	285.0

Measurements of 30 segments per treatment. Solution: 0.01 M phosphate buffer, pH 6.8 with 2 % sucrose ± riboflavin at concentrations stated. Pretreatment for 3 hrs in darkness then ± light for 1 hr. Final measurements 24 hrs after beginning of experiment.

ANALYSIS OF VARIANCE

	D.F.	MEAN SQ	
		TOTAL INCREMENT	POST-ILLUM. INCREMENT
RFN	4	14.98	6.6
Light	1	714.15 †	714.14 †
Interaction	4	2.69	2.69
Error	50	9.54	10.66

† Significance, 1 %.

period of 24 hours when the segments were remeasured and the total increment obtained.

The experiments comprised a factorial design of ± IAA ± RFN (both at 1 ppm in .01 M phosphate buffer, pH 6.8 with 2 % sucrose) ± light, and in addition a range of concentrations when using RFN alone. Six replicates (each of 5 segments) were used per treatment and the data were analysed statistically in two ways, a) using total increment and b) post-illumination increment only, by making the assumption that the increment of growth during the pretreatment period was uniform among replicates within treatments.

For convenience and also to economise material the experiments were executed at the same time. The data for the controls and RFN (1 ppm) were therefore common to the two experiments.

RESULTS: The result of one such experiment is shown in tables VII and VIII. In table VII are recorded the increments in the different concentrations of riboflavin. It will be seen that they were slightly less than the increment in the control solution, but there was neither a significant effect of riboflavin nor an interaction with light.

The post-illumination increments of the illuminated segments are of some interest because they did not differ significantly from the increment of growth of the control segments. Thus uniform growth was shown which implies uniform auxin content, so that inactivation of the native auxin by the riboflavin absorbed from the medium during pretreatment could not have taken place upon illumination. This leads to the conclusion that the applied riboflavin either did not enter the cells, or if it did, then it failed to react with the native auxin.

The result of the factorial experiment (± IAA ± RFN ± Light) is shown in table VIII. The data for total increment are directly comparable with the corresponding treatments in Galston & Baker's experiments, except that they mention no pretreatment in darkness nor do they include data on the growth of pea epicotyl segments in a medium containing riboflavin alone.

Two features call for comment: the significant effect of riboflavin and the interaction between IAA and light.

In the concentration series already discussed (table VII) riboflavin did not affect the growth of the segments, consequently the significant effect shown in this factorial experiment must be regarded as spurious. It clearly arose from a treatment not included in the concentration series and it was obviously due to the small post-illumination increment in the treatment IAA + RFN + light (261 mm) as compared with that in IAA + light (306 mm). For significance at 5 % a difference of 32 is required between the entries. Of the four comparisons involving RFN only that between RFN + IAA + light and IAA + light gives a sig-

TABLE VIII

EFFECT OF IAA, RIBOFLAVIN AND LIGHT ON THE GROWTH OF WHEAT COLEOPTILE SEGMENTS

LIGHT, FT-C	IAA + RFN	RFN	IAA	CONTROL
	Total increment, mm			
400	338.5	276.0	380.0	286.5
0	421.5	317.0	431.0	322.5
	Post-illumination increment, mm			
400	261.0	242.5	306.0	249.0
0	344.0	283.5	357.0	285.0

Measurements of 30 coleoptile segments immersed in 0.01 M phosphate buffer, pH 6.8 with 2 % sucrose ± IAA and riboflavin (1 ppm) ± light for 1 hr after 3 hrs pretreatment in darkness. Final measurement 24 hrs after beginning of experiment.

ANALYSIS OF VARIANCE

	D.F.	MEAN SQ	
		TOTAL INCREMENT	POST-ILLUM. INCREMENT
IAA	1	2836.67 ††	901.33 ††
RFN	1	93.51 †	90.75 †
Light	1	927.51 ††	927.52 ††
IAA × RFN	1	25.54	52.08
IAA × light	1	67.70 †	67.69
RFN × light	1	28.53	28.52
Triple interaction	1	15.18	15.19
Error	40	16.95	20.60

† Significance, 5 %.
†† Significance, 1 %.

nificant difference, but this is so large that it wholly overshadows the absence of significance in the other three comparisons. The small increment in this treatment was due not to the presence of RFN but to the removal of IAA, for after illumination this medium was free of IAA by photodestruction and thus contained only RFN (it will be shown later in table IX that a brief illumination did not decompose RFN). It would thus be expected that the post-illumination increment in this treatment would equal that in the medium containing RFN alone, and the values in the table (261 mm and 242.5 mm), in fact, do not differ significantly.

The interaction between IAA and light arose from the same cause, namely, the inclusion of the treatment IAA + RFN + light as such, in the appropriate computation.

The results thus show a strongly promotive effect due to IAA, a strongly inhibitory effect of light and no effect unequivocally attributable to riboflavin; first and second order interactions, upon analysis, all failed to reach significance.

On the Photolysis of IAA in Vitro

The papers of Galston and co-workers (9, 10, 11) leave little doubt that they consider riboflavin to be important in growth and phototropic phenomena as an agent causing the destruction of IAA in light. Subsequently the role of riboflavin was attributed to a flavoprotein system and peroxidase and this led to the investigation on IAA-oxidase (12, 13). The validity of the flavo-protein hypothesis has been questioned by Kenten (22) who has shown that when the very powerful IAA oxidase found in the wax-pod bean was inactivated by the removal of its prosthetic group its activity was not restored by the addition of either riboflavin, flavin mononucleotide (FMN) or flavine-adenine-dinucleotide (FAD). Further, Goldacre (16) using chromatographic techniques, has shown that the photoclastic activity of pea extracts with respect to IAA is confined to spots which do not correspond in position with riboflavin.

Further experiments were therefore carried out with a variety of flavin derivatives.

Materials and Methods: IAA solutions were used at a concentration of 25 μgm/ml, to which was added 1 ppm of riboflavin, FMN, lumiflavin (LFN), acriflavin (AFN) or picric acid. Corresponding solutions were kept in darkness or exposed continuously to white fluorescent light (100 ft-c), and aliquots were withdrawn at intervals for the determination of IAA content by the Salkowski reaction (28). The maximum reading was obtained by observing the development of the pink colour at minute intervals on a EEL colourimeter with a 625 green filter. In the tests with RFN and FMN microbiological assays were also carried out. The IAA and riboflavin used was supplied respectively by British Drug Houses Ltd. and La Roche, but lumiflavin was prepared as described by Warburg & Christian (32), and it was used at approximately 1 ppm. The concentration could not be determined as this substance does not support the growth of *Lactobacillus casei* (27).

Table IX

Photolysis of IAA in the Presence of a Variety of Sensitizers

Sensitizers	Light, ft-c	Time, hrs						
		0	0.5	1	1.5	3	18	20
IAA content as percentage of initial content								
RFN	100	100	52.8	28.3	10.5	...	2.6	...
	0	100	100	99.1	98.2	...	95.2	...
FMN	100	100	61.8	40.3	26.4	...	3.1	...
	0	100	100	99.8	99.0	...	97.4	...
LFN	100	100	...	...	...	...	...	4.4
	0	100	...	...	...	...	...	99.4
AFN	100	100	74	59.5	47.5	...	18	...
	0	100	101	101	101	...	99.3	...
Picric acid	100	100	...	99.4	...	97.9	...	...
	0	100	...	97.7	...	99.0	...	...
Flavin content, μgm/ml (initially 1.0) of the RFN and FMN solutions above								
RFN	100	1.01	0.92	0.90	0.90	...	0.15	...
	0	0.96	1.10	0.90	1.00	...	0.98	...
FMN	100	1.00	0.98	0.84	1.00	...	0.06	...
	0	1.13	1.00	...	1.07	...	0.90	...

Results: The results of these experiments are recorded in table IX in which the content of IAA is given as a percentage of the initial content, and the flavin content in μgm/ml.

These results show that LFN and FMN were almost as active as was RFN in phytolysing IAA. Picric acid was very much less effective, if at all, and AFN another yellow, fluorescent material with a structure akin to riboflavin, but unlikely to occur naturally, was also highly effective. Thus IAA appears to be inactivated in vitro by many substances (8).

Although the major part of the IAA was decomposed in 1.5 hrs, under the conditions used, the analyses show that the flavins were hardly destroyed in this time; after 18 hours, however, there was almost total decomposition.

It may also be noted that for microbiological assays FMN was just as suitable as a source of riboflavin as was this substance itself. As the response was not modified by the presence of IAA and the photodecomposition products, it would appear that *L. casei* differs in this respect from *L. arabinosus* (15).

Discussion

In addition to the difficulties noted in the introduction of interpreting phototropic reactions in terms of known effects of light on extension growth further impediments to the acceptance of the "riboflavin theory of photoperception" emerged as this work progressed.

The theory as originally stated suggests that riboflavin inactivates auxin in the light and consequently

the presence of free riboflavin in the cell is essential for the reaction to occur.

Galston and Baker (11) assayed Avena coleoptiles (the 1-mm tip and two succeeding 5-mm segments) and found a content of about 30 μgm/gm dry weight, which they regarded as free riboflavin. This cannot be so; it must be an estimate of total flavin, free and combined and this contention is supported by the finding that *L. casei* can utilize FMN. Bessey, Lowry and Love (4) have estimated the amounts of the individual nucleotides in rat retinae and they detected only FMN and FAD. Further, Goldacre (16) chromatographed pea dialysates which actively destroyed IAA in light and found that the spot where riboflavin should have occurred was "scarcely discernible." He therefore concluded that "the bulk of the activity did not correspond with the riboflavin spot." It may also be concluded that the riboflavin content must have been extremely low. The evidence, although scanty, points to the absence of free riboflavin [2] in cells, but the matter requires to be specifically demonstrated for plant material.

As FMN is known to occur in vivo as an enzyme prosthetic group its in vitro behaviour towards IAA was investigated and after it was found to inactivate IAA in the light its mobility in oat plumules was tested. It behaved exactly as did riboflavin. FAD has not yet been tested in this connection.

A difference between the in vitro and in vivo behaviour of riboflavin may also be mentioned. When riboflavin is illuminated in vitro it is decomposed, but when plants are illuminated their "riboflavin" content increases (17, 18, 19). If in vivo flavins behaved towards IAA as they do in vitro, plants exposed to continuous light could not grow. Again, in animal tissue (rat retinae) "flavin" is stable to intense and prolonged illumination (3).

Thus the reference of this in vitro reaction to the cell is not a direct and simple matter.

As already stated the initial object of this work was to infiltrate coleoptiles with riboflavin, and then to test their phototropic sensitivity. The attainment of this apparently simple objective was frustrated by the finding that riboflavin when applied in solution via the transpiration stream to such coleoptiles (for a 2-hr period) failed to occasion a measurable effect on their growth. The possibility that the applied substance was not reaching the coleoptile was thereupon examined and confirmed. Attempts to obtain coleoptiles with high riboflavin contents by allowing the seedlings to absorb it through their roots were also unsuccessful. The data presented show that riboflavin accumulated in tissues which were in contact with the solutions, but that it travelled within the plant either slowly or not at all. This phenomenon was recorded by Charles (5) who suggested that positively charged molecules of anti-biotics, which travelled slowly in the xylem, were held on the vessel walls by negative charges. As negatively charged molecules should then travel freely an attempt was made to infiltrate coleoptiles by using a riboflavin solution at pH 9.5. This too was unsuccessful.

The absence of effects on growth in these experiments suggested that RFN might not penetrate the cell and further experiments using wheat coleoptiles, also showing no effects on growth, reinforced this opinion.

It was thus impossible to reconcile these findings with Galston and Baker's results which showed that RFN was stimulatory in darkness and inhibitory in the light, for penetration and subsequent action in a metabolic sequence affecting growth is implicit in the meaning of the words "stimulatory" and "inhibitory."

Their data (11, table 1, p. 774) which are reproduced here were therefore re-examined.

IAA μGM/ML	RFN μGM/ML	GROWTH IN DARK	RELATIVE GROWTH	GROWTH IN LIGHT	RELATIVE GROWTH
0	0	0.90 ± 0.07	53	0.47 ± 0.08	32 (100)
0.1	0	1.71 ± 0.27	100	1.45 ± 0.16	100 (309)
0.1	0.1	2.02 ± 0.32	118	0.88 ± 0.07	61 (187)
0.1	1.0	2.35 ± 0.30	137	0.62 ± 0.06	43 (132)
0.1	10.0	1.02 ± 0.19	60	0.61 ± 0.06	42 (130)

The figures for relative growth in parentheses have been added to the table by the present author.

Concerning the data for growth in darkness it will be seen that, with the standard errors quoted, the means under discussion 1.71, 2.02 and 2.35 do not differ significantly (Prof. Galston informed me that there were 12 replicates in these experiments). The differences may thus be random variation and the data do not support the conclusion that riboflavin stimulates growth in darkness.

Now, with regard to the light data it will be seen that Galston and Baker have selected the increment in the presence of IAA alone as their standard of reference and on this basis the addition of 0.1 μgm RFN gives an "inhibition" of growth to 61 %. It is nevertheless evident that when the true controls are taken as 100 (the relative growth figs in parentheses) this medium *promoted* growth; there was a *net increase* of 87 %, and promotion occurred to a lesser degree in the other media containing IAA and riboflavin.

Regarded in this way Galston and Baker's data show neither a stimulation nor an inhibition and thus are not at variance with the conclusions already presented.

Thus, when tissue segments are immersed in a medium containing IAA and riboflavin the probable sequence of events is as follows. Both IAA and riboflavin penetrate the tissues but only IAA enters the cells and growth of the segments consequently begins. Riboflavin has no effect on this elongation. Upon illumination two effects take place, the one directly upon the tissues which results in a growth inhibition

[2] Prof. Galston has drawn my attention to publications by H. Kondo (Jour. Agr. Chem. Soc. Japan 30: 393–397 and 690–696) in which the occurrence of free riboflavin in soya beans is reported.

not related to the presence of the applied riboflavin; the second upon the solution in that the residual IAA is inactivated in the presence of the residual riboflavin, which, however, remains unaffected (table IX). Subsequently in darkness growth of the segments proceeds due to the continued presence of the native auxin. These segments, however, grow less than similar ones in a medium containing IAA but no riboflavin because here the IAA remaining in the solution at the end of the pretreatment period is not completely inactivated during illumination (14) and it is thus available during the post-illumination period. This explanation accounts for the fact that the *post-illumination* increments in the control, RFN, and IAA + RFN solutions are not significantly different from each other, while those in IAA alone do differ significantly from the controls (table VIII).

It will now be apparent that Galston & Baker have employed "inhibition" to mean the reduction of IAA induced growth by the in vitro inactivation of the applied IAA. This is an unusual meaning. An inhibition usually means an extension less than that shown by the controls due to a reaction in vivo between the inhibitor and a metabolite intimately concerned with a growth process. The absence from their paper of data on the behaviour of pea segments in a medium containing riboflavin alone prevented them from detecting an effect in this sense. But in the conclusion drawn from the pea segment experiment, already quoted in the introduction, "inhibition" conveys its usual meaning for entry and interaction with a growth hormone in vivo are clearly implied. This conclusion must therefore be considered unproven.

Their paper thus presents further examples of *in vitro* reactions between IAA and RFN and the statement in the summary (and discussion) "By means of *in vivo* and *in vitro* experiments it has been established that riboflavin is a photoreceptor in the destruction of auxin by visible light" cannot now be maintained.

The demonstration of an interaction between applied riboflavin and native hormone in vivo is an essential prerequisite for the establishment of a theory of photoperception and all attempts to display this effect have failed. Consequently Galston & Baker's claim to have established the theory does not carry conviction.

The hypothesis that native flavin and native auxin interact in the light in vivo as suggested by Galston (9) has not been excluded by this investigation, but no evidence to support it has yet been found in the literature.

Summary

In order to test the "riboflavin theory of photoperception" proposed by Galston and co-workers factorial experiments were carried out with plumules of oat seedlings which were severed close to the seed. These plumules were then supplied with combinations of riboflavin and IAA in solution via the transpiration stream.

No effect of riboflavin on the growth of the coleoptile was observed nor any interaction with either light or IAA. The flavin content of the plumules was estimated after treatment using the Lactobacillus casei assay method, and it was found that after a 2-hour pretreatment period in the riboflavin solution, the flavin content of the coleoptile was unchanged, whereas that of the immersed tissue was very high. After a 24-hour pretreatment the applied riboflavin had reached the coleoptile.

As the exposure to light was given after a 2-hour pretreatment in darkness the absence of response was accounted for. Illumination after a 24-hour pretreatment also showed neither an effect of riboflavin, nor an interaction with light. In this experiment riboflavin was known to be present in the coleoptile and the absence of effect must have been due either to a failure of riboflavin to enter the cells or to the absence of a reaction with the naturally occurring auxin.

Efforts to increase the riboflavin content of the coleoptiles of intact seedlings by growing them for long periods in culture media containing riboflavin also failed as assays showed no entry into the plumule but high concentrations in the roots.

Further factorial experiments were therefore carried out using segments of wheat coleoptiles immersed in solutions containing combinations of IAA and RFN. The overall effect of IAA was to promote growth, and of light very strongly to depress it, while riboflavin had no effect at all. Neither 1st nor 2nd order interaction of these factors reached significance.

Experiments on the photolysis of IAA in vitro showed that flavin mononucleotide, acriflavin and lumiflavin decompose IAA in the light almost as effectively as does riboflavin.

RFN and FMN and the flavin content of plant extracts were assayed using *Lactobacillus casei*. The values obtained for the plant extracts must refer to total flavin content and not to the content of free riboflavin.

In view of these results Galston and Baker's data were re-examined and shown to offer no evidence for their suggestions that riboflavin in the presence of IAA occasions a promotion of growth in darkness, and an inhibition of growth in the light. The "inhibition" in light is shown to be a reduced growth promotion. Their claim to have established the theory is discussed.

The author gladly acknowledges the encouragement and guidance given by Professor F. G. Gregory, F.R.S. Thanks are also due to R. E. Weston (Department of the Government Chemist) for the cultures of *Lactobacillus casei* and for advice on microbiological assays: to Dr. E. H. Lloyd (Department of Mathematics, Imperial College) for assistance with the analyses of covariance; to Dr. W. G. Kenner and Mr. C. B. Reese (Department of Chemistry, Cambridge) for the gift of a pure sample of FMN (virtually riboflavin-free) and to Miss M. K. Baker for technical assistance during the course of the work.

Prior to publication this paper was submitted for criticism to Professor A. W. Galston who pointed out two errors, one of fact and the other of interpretation. These have been corrected and I thank him for his co-operation in this connection.

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PARTICIPATION OF CYTOCHROMES IN THE RESPIRATION OF THE AROID SPADIX[1,2]

CONRAD S. YOCUM[3] AND DAVID P. HACKETT[4]

THE BIOLOGICAL LABORATORIES, HARVARD UNIVERSITY, CAMBRIDGE 38, MASSACHUSETTS

Although a cyanide-resistant fraction of respiration is present in many plant and animal tissues, the mechanism of terminal oxidation in such respiration has never been completely clarified. On the basis of their study of the rapid, cyanide-resistant respiration of the Aroid spadix, James and Beevers (8) proposed that flavoproteins serve as terminal oxidases in this tissue. Their conclusion was based in part on the finding that the measured respiratory rate was strongly dependent upon the partial pressure of oxygen. In addition, they found no spectroscopic or enzymatic evidence for the presence of a cytochrome system.

The rapid respiration of the Aroid spadix has been reinvestigated here. A relative insensitivity to cyanide (HCN) and carbon monoxide (CO) has been confirmed. With an adequate rate of oxygen supply, the tissue exhibits a very high affinity for oxygen. This observation led to a spectroscopic and enzymatic search for cytochrome components. The results reported here demonstrate the participation of cytochromes in the respiration of the Aroid spadix. A preliminary report of the results was given earlier (23).

MATERIALS AND METHODS

Flowers of *Philodendron grandifolium* and *Peltandra virginica* were obtained from the greenhouse at Smith College, Northampton, Massachusetts, and from local swamps, respectively. For measurements of respiration, sections 0.25 to 1 mm thick were sliced from the male portion of the spadix. Oxygen exchange at atmospheric pressure was measured either by conventional manometry, with slices suspended in buffer, or by volumetry (21) for slices in a moist gas phase. Oxygen exchange at 7.8 atmospheres was also measured volumetrically. A clear cellulose acetate compensating chamber encased an experimental and a blank volumeter of equal dimensions (21). HCN was supplied from $Ca(CN)_2$–$Ca(OH)_2$ mixtures as described by Robbie (19). Gas mixtures were made up from commercial tanks of O_2, N_2, and CO (scrubbed with alkali).

To obtain the particulate preparation, male flowers were ground in a small volume of 0.05 M phosphate buffer, pH 7, which was also 0.3 M with respect to mannitol. The homogenate was transferred to a centrifuge tube and spun at $1{,}000 \times g$ for 5 minutes. The supernatant was then centrifuged at $10{,}000 \times g$ for 15 minutes, and the residue suspended in 1 ml of the original grinding medium to give the final particulate preparation. All of these operations were carried out at 0° C. Cytochrome c oxidase activity was assayed in a Beckman DU spectrophotometer by following the decrease in optical density (OD) at 550 mm. Cytochrome c obtained from Sigma was reduced with $Na_2S_2O_4$ and the excess hydrosulfite removed by aeration.

Difference spectra were computed from the light transmission through about 1 cm (= 1.5 Peltandra spadix diameters) of tissue in a moist gas phase. A DC single beam spectrophotometer was operated manually as follows: wavelength band-width 3.3 mμ, "noise" about 10^{-3} OD units, drift 10^{-3} OD units per minute, galvanometer period 15 seconds. The light source was a 6-volt 18-amp ribbon filament lamp fed from a storage battery, which was charged at the rate of discharge. The monochromator was a Bausch and Lomb 500-mm grating type with 600 grooves/mm. A photomultiplier tube (RCA 6217), operated at 700 volts, received the transmitted light. The voltage drop across a 110-megohm load resistor was converted to galvanometer deflections through a cathode follower bridge (6). Light minus dark deflections were first recorded at a series of wavelengths for the tissue in N_2. After flushing with O_2, the series was repeated in the same sequence. A similar sequence of measurements was then made on the same tissue in the presence of HCN.

RESULTS

RESPIRATORY STUDIES: Table I shows that 10^{-4} M and 10^{-3} M HCN stimulate the respiration of Philodendron slices maintained in a moist gas phase. Similar experiments show (table II) that this tissue also respires more rapidly in a gas mixture of 95 : 5 CO : O_2 than in 95 : 5 N_2 : O_2. From the data of table II it is evident that the respiratory rate in 5 % O_2 nearly equals that in air (21 % O_2). This is in marked contrast to the observation on *Arum maculatum* (8), where approximately 75 % inhibition of the observed

TABLE I

CYANIDE PROMOTION AND INHIBITION OF PHILODENDRON SPADIX RESPIRATION IN VOLUMETRIC RESPIROMETERS

HCN	RESPIRATORY MEASUREMENTS	
	A	B
$\times 10^{-4}$ *M*	*μl* $O_2 \times$ *gm fresh wt*$^{-1} \times$ *hr*$^{-1}$ *at 25° C*	
0	1320	970
1	1620	1420
10	1700	1540
40	1160	....

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[2] This work was supported in part by grants from the American Cancer Society to Dr. K. V. Thimann and to Harvard University.

[3] Present address: Department of Botany, Cornell University, Ithaca, New York.

[4] Present address: Department of Biology, University of Buffalo, Buffalo, New York.

TABLE II

CARBON MONOXIDE (CO) PROMOTION OF PHILODENDRON SPADIX RESPIRATION IN VOLUMETRIC RESPIROMETERS

AIR	$N_2 : O_2$ 95 : 5	$CO : O_2$ 95 : 5
	$\mu l\ O_2 \times gm\ fresh\ wt^{-1} \times hr^{-1}$ at 25° C	
1280	1060	1490
1710	1380	1730
1970	1540	2040

rate resulted from reduction of the oxygen partial pressure from 21 to 5 %.

Since both the plant material and the methods differed in the two cases, the dependence of the respiratory rate of Philodendron slices on oxygen pressure (pO_2) was determined by the two methods and the results compared. Slices respiring in a liquid phase in a Warburg vessel, under conditions approximating those of James and Beevers, show a marked dependence upon pO_2, as shown in figure 1 (curve B); 0.15 atm O_2 supports half the maximal rate. On the other hand, if no external liquid is in contact with the slices the rate of respiration depends upon the pO_2 only at very low pressures (fig 1, curve A). Under these conditions, the pO_2^{50} (O_2 partial pressure required for half the maximal respiratory rate) is 0.002 atm O_2. Thus, the removal of the external liquid (essentially water in this case) from the respiring spadix tissue decreases the oxygen pressure required for half saturation about 100 fold.

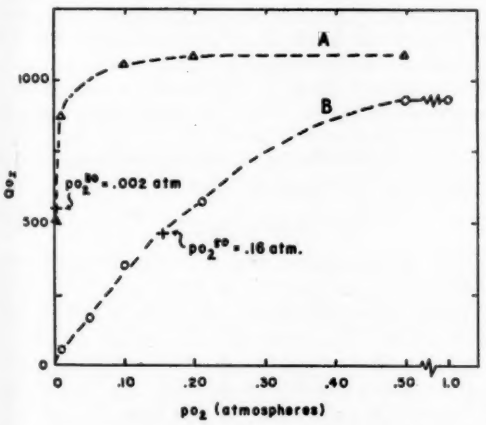


FIG. 1. Respiratory rate Q_{O_2} in $\mu l\ O_2 \times gm$ fresh $wt^{-1} \times hr^{-1}$ at 25° C of *Philodendron grandifolium* spadix slices as a function of the oxygen partial pressure, pO_2. The data of curve B were determined manometrically in 15-ml vessels shaken at 120 oscillations (4 cm) per minute; 0.4 gm of tissue was suspended in 3.0 ml of 0.06 M phosphate buffer, pH 7. The data of curve A were determined volumetrically in 4-ml vessels containing 0.2 gm tissue in contact with no external liquid phase. All data were from the same spadix.

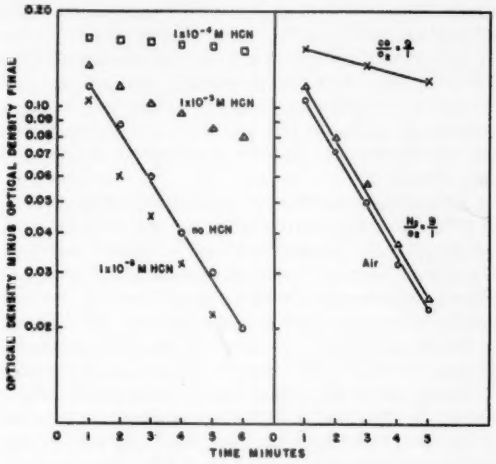


FIG. 2. Cytochrome c oxidase activity of a particulate preparation from Peltandra spadix. Final concentrations in cuvette: mannitol, 0.1 M, phosphate buffer, 0.05 M, pH 7, cytochrome c, 2×10^{-5} M. The reaction was initiated by introducing 0.25 ml of the particulate suspension prepared from 0.050 gm of spadix. The total volume was 3.25 ml and the temperature 25° C.

Experiments of the type illustrated in figure 1, curve A, were also carried out to test the effect of CO on the oxygen affinity. The results, shown in table III, demonstrate that the affinity is still high; e.g., pO_2^{50} is attained at 0.004 atm by Philodendron at 25° C. This value may be compared to the pO_2^{50} of 0.002 atm measured in the absence of CO (fig 1).

CYTOCHROME C OXIDASE: Peltandra spadix particles ($10{,}000 \times g \times 15$ min fraction) contain an active cytochrome c oxidase as shown in figure 2. First order kinetics are followed approximately; a velocity constant, k, of 0.36 to $0.38 \times min^{-1}$ was estimated for this preparation. The initial rate of cytochrome oxidation ($k \times$ initial concentration of 2×10^{-5} M) corresponds to an O_2 uptake of 2.42 $\mu l \times hr^{-1} \times ml^{-1}$. This times the dilution (0.015^{-1}) gives 161 $\mu l \times hr^{-1} \times grams^{-1}$ for the computed respiratory rate of the spadix which can proceed via recovered particulate cytochrome c oxidase. Assuming that approximately 10 % of the activity of this enzyme is recovered during preparation, the computed rate of the spadix respiration be-

TABLE III

THE PARTIAL PRESSURE OF O_2 REQUIRED FOR HALF MAXIMAL RESPIRATORY RATE (pO_2^{50}) OF AROID SPADIX TISSUES RESPIRING IN CARBON MONOXIDE (CO)

PLANT	TEMP, ° C	pCO,* ATM	pO_2^{50}, ATM
Philodendron	25	0.996	0.004
Peltandra	15	7.8	0.012
Peltandra	0	0.998	0.002

* pCO = partial pressure of CO.

comes 1600 $\mu l \times hr^{-1} \times grams^{-1}$. The measured spadix respiratory rate is of this order of magnitude.

Also shown in figure 2 are experiments with inhibitors. 1×10^{-5} M HCN suppressed the rate of cytochrome c oxidation by more than one half. A gas mixture of 0.90 atm CO and 0.10 atm O_2 decreased the rate constant to 23 % of that in 0.90 atm N_2 and 0.10 atm O_2.

SPECTROPHOTOMETRY: A tissue difference spectrum, i.e., optical density in the reduced state minus that in the steady state oxidized, is shown in figure 3, curve A. Absorption maxima occur at approximately 605 and 550 mμ, which correspond to the α-bands of reduced cytochromes $a + a_3$ and c, respectively. The 550 mμ maximum is larger by a factor of about 2 and shows asymmetry on the long wavelength side, probably due to absorption by the α-band of a cytochrome b. These results, which show the reduction of these components in N_2 and their oxidation (at least partial) in O_2, strongly suggest the participation of cytochromes in the respiration of this tissue.

The difference spectrum was also determined in a concentration of HCN which does not inhibit respiration (see table I) yet inhibits isolated cytochrome oxidase by about 95 %. As shown in figure 3, curve B, the absorption maxima at 550 and 605 mμ are decreased by about one half and another maximum at 560 mμ (here assumed to be cytochrome b) is evident. There is a suggestion of another absorption band at 585 to 590 mμ. It is evident that respiration in the presence of cyanide is mediated, at least in part, by cytochrome components. However, the relative contributions of the individual cytochromes, as revealed by the difference spectra, are markedly altered by cyanide.

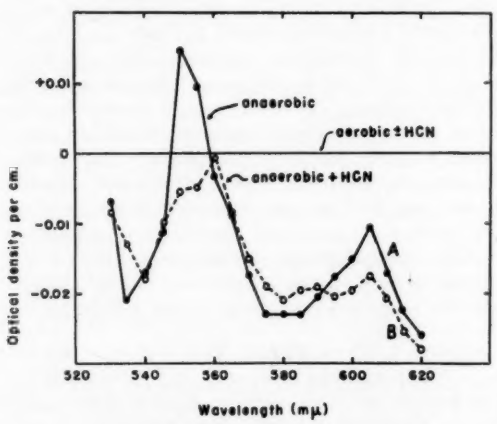


FIG. 3. Difference spectra (reduced minus steady state oxidized) for the spadix of *Peltandra virginica*. Each point for the oxidized state was obtained about 5 min after the corresponding one for the reduced state. HCN (2×10^{-4} M) was furnished by KCN–KOH buffers (19) in the gas train and on filter paper in the light absorption cell.

TABLE IV

OPTICAL DENSITIES, CONCENTRATIONS AND TURNOVER NUMBERS OF CYTOCHROMES IN A RESPIRING PELTANDRA SPADIX

MEASUREMENTS	CONC OF HCN, M	CYTOCHROMES		
		c	b	$a + a_3$
OD (cm^{-1})	0	0.031	0.020	0.012
Conc (μM)	0	1.7	1.1	0.7
TN (sec^{-1})	0	30	46	73
OD (cm^{-1})	2×10^{-4}	0.012	0.020	0.007

The optical density (OD) data were obtained from the difference spectra of figure 2 by subtracting the OD at a neutral reference wavelength from that at the absorption maximum: cytochrome c (550 to 540 mμ), b (560 to 580 mμ) and $a + a_3$ (605 to 615 mμ). An extinction coefficient of $18 \times mM^{-1} \times cm^{-1}$ was used for each component (2). For the turnover (TN) estimation, a respiratory rate of 1000 $\mu l\ O_2 \times gm$ fresh $wt^{-1} \times hr^{-1}$ was assumed.

Of the 6 or so difference spectra obtained by this method, 4 showed the 605 mμ peak below the base line. The pair of curves shown on figure 3 were the only two that were obtained on the *same* spadix and that included the cytochrome a region. The coincidence of the two curves (no corrections were applied to either curve) is evidence at least of their reproducibility within a single spadix. Also when several of the wavelength points were remeasured at the end of a run, they could be reproduced within 10^{-3} OD units: that is the spectrophotometer did not drift excessively.

The concentrations of participating cytochromes, estimated by Chance's method (2) from the difference spectra of figure 3, are shown in table IV. In contrast to the cytochrome $a + a_3$ and c values, which are decreased by HCN, the cytochrome b value showed no detectable change. That the steady state oxidized level of cytochrome b is not diminished by HCN points to its functioning in respiration both in the presence and absence of the inhibitor.

DISCUSSION

The studies reported here show that measured respiratory rates at low oxygen pressures are strongly dependent upon the method of oxygen supply to the Aroid spadix. When furnished from a moist gas phase, a high O_2 affinity is observed. If, however, a water layer separates this rapidly respiring tissue from the gas phase, the apparent affinity is lower by a factor of about 100. We attribute such differences to the fact that the diffusion coefficient of oxygen in water is 3×10^{-5} times its value in air at 20° C (9). From this, it follows that diffusion through the liquid suspending medium also limited the rapid rate of O_2 exchange in the earlier experiments with *Arum maculatum* (8). That this is indeed so has now been confirmed by Simon's (personal communication) demonstration of a high oxygen affinity for *Arum maculatum* slices respiring in a moist gas phase.

Several types of evidence for the participation of cytochromes in the respiration of the spadix have been provided here. First, a comparison of the oxygen affinity ($1/O_2^{50}$) of the intact tissue with the values for a variety of isolated oxidases (table V) reveals that it exceeds the affinity of all except cytochrome oxidase. This fact alone argues strongly against the functioning of copper- or flavin-containing oxidases in respiration. The oxygen affinity for the spadix (3×10^5) equals that of freshly cut potato tuber slices, in which at least 70 % of the respiration is mediated by cytochrome oxidase (21). On the other hand, the values for yeast (1×10^6) and Aerobacter (3×10^7), both of which utilize a cytochrome system, are higher. We attribute this difference to longer oxygen diffusion paths within the tissues than within the microorganisms.

The second type of evidence is the demonstration of cytochrome c oxidase in extracts of Philodendron and Peltandra. Both with respect to its inhibitor sensitivity (fig 2) and its intracellular localization (on particles), the spadix enzyme is similar to the cytochrome oxidase isolated from other cells. This activity is generally ascribed to cytochromes $a + a_3$, suggesting that these components are present. The enzyme is probably present in all spadices, since it has also been demonstrated in *Symplocarpus foetidus* (1, 7). However, this evidence alone does not prove the participation of cytochrome oxidase in the respiration of intact tissues.

Further evidence has been supplied by spectrophotometric observations on intact tissues. The difference spectrum (fig 3) shows that cytochromes b and c, as well as cytochrome oxidase, are involved in the reaction with molecular oxygen. The concentrations of the cytochromes functioning in respiration, as computed from the optical density differences (fig 2) and the extinction coefficients are included in table IV. Both the absolute ($\sim 10^{-6}$ M) and relative amounts are similar to those reported for other aerobic cells (2, 13). From these data and the spadix respiratory rate, turnover numbers were estimated for the cytochromes $a + a_3$, b, and c. The turnover numbers are within the range of published values of 10 to 100/ sec for yeast, muscle and roots (2, 13). Thus a respiratory chain of the $b \rightarrow c \rightarrow a + a_3$ type can account for the spadix respiration. In spite of this evidence that the Aroid spadix contains and uses a complete cytochrome system, the tissue respiration shows a remarkable insensitivity to cytochrome oxidase inhibitors, and this observation remains to be explained.

Several possible causes of this insensitivity to inhibitors are considered: 1) The oxidation of CO to CO_2, with a consequent uptake of oxygen, could mask a respiratory inhibition. This possibility has been eliminated for CO-insensitive potato slices (21) and mature leaves (4) and it is not suggested for the Aroid spadix, since the volume of gas consumed never exceeded the initial volume of oxygen. 2) The oxygen-activating system might be protected in some manner from the inhibitors. Evidence that CO and cyanide do in fact combine with some respiratory component is provided by the observed stimulation of respiration (tables I, II) and by the alteration in the difference spectrum induced by cyanide (fig 3). 3) The presence of a large excess of cytochrome oxidase relative to the rate limiting step in respiration could account for the tissue's insensitivity to HCN and CO. This hypothesis has been advanced recently to account for the case of mature leaves (5). The quantity that would be required for the Aroid spadix respiring in CO may be computed by assuming that (a) all electrons are transferred to cytochrome c oxidase (cytochrome a_3) and (b) the following modification of Warburg's partition equation describes the CO inhibition of respiration:

$$\frac{n}{(a_3) - n} \cdot \frac{(CO)}{(O_2)} = K$$

where

n = residual quantity of the oxidase, relative to an amount 1 required for the respiratory rate in air.
(a_3) = total quantity of the oxidase, relative to an amount 1 required for the respiratory rate in air.
(CO) = CO partial pressure.
(O_2) = O_2 partial pressure.
K = relative affinity constant of the oxidase for O_2 and CO.

K was computed from 77 % inhibition of Peltandra cytochrome c oxidase preparation by a 9/1 ratio of CO/O_2 (fig 2) and found to be 2.7. This is in close agreement with Winzler's value of 2.5 from yeast respiration experiments (22).

TABLE V

OXYGEN AFFINITIES ($1/K_m$ OR $1/[O_2]^{50}$) * FOR RESPIRATION AND OXIDASES

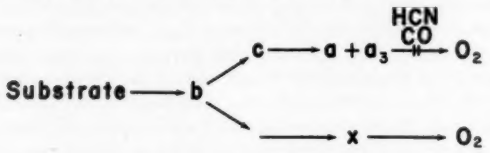
	TEMP, °C	$[O_2]^{50}$	$1/[O_2]^{50}$ OR $1/K_m$	REF
Respiration				
1. Aerobacter	19	3.1×10^{-8}	3.2×10^7	(12)
2. Yeast	20	1×10^{-6}	1×10^6	(22)
3. Potato (tuber slices)	25	3×10^{-6}	3×10^5	(21)
4. Aroid spadix	25	3×10^{-6}	3×10^5	(fig 1)
Oxidases				
1. Cytochrome c	20	2.4×10^{-8}	4×10^7	(12)
2. Ascorbic acid	30	3×10^{-4}	3×10^3	(15)
3. Ascorbic acid	25	1.5×10^{-4}	6×10^3	(21)
4. Phenol	25	3×10^{-5}	3×10^4	(10)
5. Amino acid	38	5×10^{-5}	2×10^4	(11)
6. Xanthine	38	7×10^{-5}	1.4×10^4	(11)
7. Glucose (notatin)	38	1×10^{-4}	1×10^4	(11)

$[O_2]^{50}$ is the oxygen concentration, in moles per liter, required for half the maximum rate of oxygen uptake.

$$[O_2]^{50} = \frac{pO_2^{50} \times \alpha O_2}{22.4 \text{ liter atm/mole}}$$

where: pO_2^{50} is the partial pressure, in atmospheres, of oxygen required for half the maximum rate of oxygen uptake. αO_2 is the oxygen solubility coefficient.

From the CO/O_2 ratio (table III) required to inhibit the respiratory rate by 50 %, i.e., when n = 0.5, (a_3) was found to lie between 47 and 129 ($\sim$ 100). The oxidase turnover number resulting from $\sim$ 100 fold excess over the rate limiting step would be $\sim 100^{-1}$ that in cells where there is evidence for no large excess (yeast, e.g., 22). However, the measured spadix oxidase turnover number of 73 (sec^{-1}) is in close agreement with that of yeast (2). This suggests an activity not large enough, in relation to the rate limiting step, to support the excess hypothesis. 4) Another possibility is that this tissue possesses an alternate, cyanide-resistant pathway of electron transfer to oxygen. Such a proposal was first made by Okunuki (17) for pollen, as early as 1939. Positive evidence that this is the case in the Aroid spadix can be obtained from the spectrophotometric observations (fig 3). The rate of oxidation of cytochrome b, in contrast to c and $a + a_3$, is not suppressed by 2×10^{-4} M HCN. This finding is consistent with the following scheme of electron transport pathways:



We have assumed a single cytochrome b and a series transfer of electrons among the components wherever possible. In the presence of inhibitor, some of the electrons proceed via an alternate pathway, which includes cytochrome b and possibly another oxidase, designated here as x. The remainder of the electrons are transferred to oxygen via the partially inhibited $c \rightarrow a + a_3 \rightarrow O_2$ system. The rates of transfer through the two systems will depend on the amount of enzyme not combined with inhibitor and the rate of reduction of the oxidases. The possibility that an inhibitor alters the system which it is being used to study suggests a situation of experimental "indeterminacy."

Although the nature of component x in the above sequence is not known, some of the properties can be deduced. This component, which has little or no affinity for HCN and CO, shows a high affinity for oxygen ($1/[O_2]^{50}$ in CO = 1.5×10^5 M) and is able to mediate a relatively rapid rate of oxidation. The simplest possibility would be that cytochrome b is oxidized directly by oxygen; the cytochromes of the b group are, in fact, autoxidizable substances which do not combine with cyanide or CO. The spectrophotometric method employed here does not exclude the possibility of other functional cytochromes of the b class. However, low temperature (–196° C) spectroscopy has resolved only one component with a band absorbing in the wavelength region of cytochromes b (1). In a study carried out independently from the present work, Lundegårdh (13) demonstrated spectrophotometrically that the cytochrome b of roots remains partially oxidized in aerated cyanide, and he has suggested that "the autoxidation of cytochrome b could possibly act as a substitute for cytochrome oxidase in the living cell." Nevertheless, the rapidity of the oxidation, turnover number = 45/sec, suggests that an additional component, above the potential of cytochrome b, functions as an oxidase in this system. By analogy with the classical system, one might expect that a member of the cytochrome a family, with a low affinity for CO and HCN, might serve as the terminal oxidase. The presence of an absorption band of 590 $m\mu$ in the difference spectrum in cyanide suggests the possible functioning of an a-type cytochrome. A band at approximately the same wavelength is also observed at – 196° C in reduced Symplocarpus particles in the absence of HCN (1).

The cytochromes b_5 and b_3 have been proposed as members of cyanide-resistant respiratory pathways in animal (18) and plant (16) cells respectively. But these hypotheses are not supported by the following evidence. The turnover number for cytochrome b_5 of 0.13/sec. (3) and an estimate computed from the data of Martin and Morton (16) of 0.05 for cytochrome b_3 are insufficient, being $\geqq 300^{-1}$ times the 45/sec computed here for cytochrome b of the Aroid spadix. In addition, cytochromes b_3 and b_5 are localized on microsomes, particles of too low oxidase activity to account for the respiration of the tissue from which they were isolated (7, 20).

It is of considerable interest to know whether electron transfer through an alternate pathway is coupled to oxidative phosphorylation. The increase in respiration caused by CO and cyanide could be the result of an "uncoupling" of phosphorylation from the normal respiration, and the activation of a non-phosphorylating electron transport system; oxidation via the latter pathway would not be limited by the supply of phosphate acceptors.

A cyanide and/or CO-insensitive respiration has been demonstrated with a wide variety of plants, animals, and microorganisms. In many, although not all, of these cases it has also been shown that the cells contain cytochrome components, including cytochrome oxidase. On the basis of our own experiments, it is suggested that the "normal" respiration of such cells may be mediated by a classical-type cytochrome system and that an alternate pathway may be activated in the presence of oxidase inhibitors, such as HCN and CO.

Summary

The following evidence is presented for the participation of cytochromes in the respiration of the Aroid spadix:

(a) The spadix respires with a high affinity for oxygen, provided the rate of oxygen supply is adequate.

(b) A particle-bound cytochrome oxidase with high affinity for cyanide and carbon monoxide can be demonstrated.

(c) Different spectra of the intact spadix show absorption maxima at wavelengths corresponding to the α-bands of cytochromes $a + a_3$, b

and c. The molar concentration of each is of the order of 10^{-6}.

(d) Turnover numbers of 10 to 10^2/sec, estimated from cytochrome concentration and respiratory rate, fall within the published values for cytochromes from yeast, muscle, and roots.

The cyanide and carbon monoxide-resistant respiration of the Aroid spadix can be accounted for by the oxidation of a cytochrome b, either directly by oxygen or via an oxidase without appreciable affinity for these inhibitors.

Since the completion of this work, the paper of D. S. Bendall and R. Hill, on "Cytochrome components in the spadix of *Arum maculatum*" (New Phytologist 55: 206–212. 1956) has come to our attention. In addition to cytochromes a, b, and c, the particulate fraction was shown to contain a component cytochrome b_7, which is rapidly oxidized even in 10^{-3} M cyanide.

We are indebted to Dr. K. V. Thimann for encouragement throughout the course of this work, and to Dr. W. D. Bonner, Jr., for the use of a spectrophotometer and a refrigerated centrifuge.

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GROWTH STIMULATION OF AVENA COLEOPTILES[1,2,3]

DONALD H. SLOCUM[4] AND JOHN E. LITTLE

DEPARTMENT OF AGRICULTURAL BIOCHEMISTRY, UNIVERSITY OF VERMONT, BURLINGTON, VERMONT

In 1941 Commoner and Thimann (1) reported the promotion of growth of oat coleoptiles by iodoacetate in the presence of sucrose and indoleacetic acid[5] and commented that "such promotion by low concentrations of an inhibitor is a very common phenomenon." The reproducibility of this effect under closely controlled conditions was established (2), and later Thimann and Bonner (3) noted that 1-amino-2-naphthol-4-sulfonic acid, an inhibitor of pyruvic carboxylase, also stimulated coleoptile elongation and suggested that this stimulation was due to conservation of pyruvate for more constructive processes.

It is the purpose of this communication to reexamine and extend this work in the light of new information about the cofactors involved with the oxidative decarboxylation of pyruvate and of α-ketoglutarate.

Additional impetus to this approach was obtained from the work of Barron and Singer (4), who, in studying the relative sensitivity of nine different sulfhydryl-containing enzymes to various inhibitors, found that "of all the enzyme systems studied, pyruvate oxidase and the enzyme for pyruvate condensation were the most sensitive to the action of inhibitors."

The conservation of pyruvate has also been found by Calvin (5) to be essential for the maintenance of photosynthetic growth of algae. It appears that light not only supplies the energy necessary for photosynthesis but also minimizes the flow of growth-essential carbon structures into the Krebs cycle. Calvin attributes this to an inhibition of pyruvate oxidation brought about by a light-activated reduction of lipoic acid, an essential coenzyme for this oxidation. It is of interest that the reduced form of this coenzyme is a dimercaptan and thus susceptible to reagents reacting with sulfhydryls.

MATERIALS AND METHODS

Deglumed oats (Ajax) were grown in the usual manner for 64 hours on moist filter paper in a dark humid room at 25° C. Seeds were not soaked before planting nor exposed to red light. From each seedling one 6-mm coleoptile section was obtained by excision with a double-bladed cutting tool 2 mm from the apical end. The sections were pooled and ten randomly selected sections transferred to each Petri dish containing 25 ml of a test solution. The test solutions consisted of basal medium and specific additives of known concentrations.

After a 24-hour incubation in the dark, the sections were measured on a Quebec Colony Counter equipped with a millimeter scale. Controls were run daily.

The basal medium contained 1 % sucrose, 2×10^{-3} M KCl 5×10^{-4} M $MnSO_4$ and 0.01 M phosphate buffer at pH 5.0. Indoleacetic acid at a concentration of 5.7×10^{-6} M (1 ppm) was used except in designated cases.

All results were based upon elongation measurements in at least two trials, unless otherwise indicated, each trial involving a minimum of 40 coleoptile sections. Statistical analysis of the data demonstrated that a growth stimulation (increase in mean length) of 8 % or better had a significance level of 1 % when 40 sections were used, while a 4 % increase was significant at the 5 % level. Growth effects of 3 % or less were considered to be without significance.

RESULTS

Growth response to auxin alone was first determined, and a typical curve was obtained. Although the elongation was greatest at 5.0×10^{-5} M auxin,

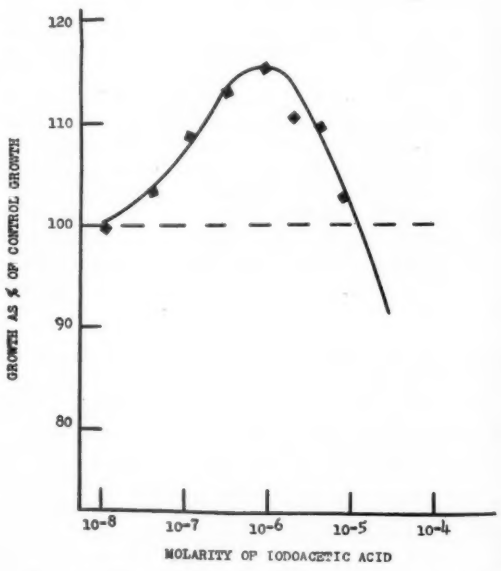

FIG. 1. Effect of low concentrations of iodoacetic acid on the growth of Avena coleoptile sections in the presence of 1 ppm IAA.

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[3] Vermont Agricultural Experiment Station Journal Series Paper Number 65.

[4] Present address: Department of Agricultural Biochemistry, The Ohio State University, Columbus 10, Ohio.

[5] The following abbreviations are used in sections of this paper: IAA, indoleacetic acid; MTA, 8-methyl thioctic acid; Co A, coenzyme A; BAL, British anti-lewisite, 2,3-dimercaptopropanol-1; CMBA, *p*-chloromercuribenzoic acid; ATP, adenosine triphosphate; GSH, glutathione; GSSG, oxidized glutathione.

5.7×10^{-6} M was used in our experiments to conform with the earlier work of others.

STIMULATION BY IODOACETATE: The stimulatory effect of iodoacetate was now studied, and the resulting curve (fig 1) was very similar to those obtained by Thimann (2). The stimulatory range appeared somewhat larger, and the percentage stimulation was considerably less. We believe this latter difference to be due to our employment of 6-mm coleoptile sections. A concentration of 1×10^{-6} M was found to produce the greatest acceleration of growth, and this concentration was employed in producing the stimulation in subsequent experiments.

The absolute increase in the control varied from day to day, but the percentage stimulation of the iodoacetate-treated sections was readily reproducible (table I).

EFFECT OF ADDED LIPOIC ACID: Since it appeared possible that iodoacetic acid was reacting with one or more sulfhydryl groups of the pyruvate oxidase system, an attempt was made to reverse the stimulation with lipoic acid. A concentration of 4.9×10^{-6} M lipoic acid of this compound which by itself exhibited no significant effect upon coleoptile growth completely reversed the stimulation due to iodoacetate (table II).

This data seemed to indicate that iodoacetate was enhancing growth by reacting with lipoic acid and thereby inhibiting the oxidative decarboxylation of either pyruvate or α–ketoglutarate. The possibility remained, however, that the added lipoic acid was simply decreasing the quantity of effective iodoacetate by reacting with it outside of the cell. Such a reaction is not very likely, since the lipoic acid was added in the oxidized or disulfide form. However, the added lipoic acid might have been reduced by the cell prior to such a combination with iodoacetate, and therefore an attempt was made to cancel the stimulation with several other sulfur-containing compounds. These results are also summarized in table II. Coenzyme A, cystine, cysteine, GSH, GSSG, BAL, thioglycollate and MTA were all ineffective in bringing about a reversal. The failure of this last compound, MTA, to effect a reversal of the stimulation is of particular interest because of its close structural relationship to lipoic acid. Presumably, since it does not replace lipoate as a coenzyme, it is not reduced by the cell and hence does not combine with iodoacetate. The additive stimulation of GSH or GSSG and iodoacetate is interesting. When such a double stimulation was obtained the growth contribution of the iodoacetic acid was approximately cancelled by the addition of lipoic acid while that due to GSH or GSSG was not (table III). We have been unable to explain this behavior.

STIMULATION BY OTHER INHIBITORS: We have found that lipoic acid will also reverse the stimulation brought about by low concentrations of other inhibitors. In 1949 Thimann and Bonner (6) reported stimulation of Avena coleoptiles by parachloromercuribenzoic acid (CMBA). This compound has a highly specific affinity for sulfhydryl groups (7). In our experiments a maximum stimulation of 12 % was obtained with this compound at a concentration of 1×10^{-6} M and a complete reversal was affected by 4.9×10^{-6} M lipoic acid (fig 2). On the other hand.

TABLE I

STIMULATION OF COLEOPTILE SECTIONS BY IODOACETIC ACID

EXPT NO.	TREATMENT	MEAN INCREASE IN GROWTH, MM/SECTION *	% INCREASE
I	Control	13.8	
	Iodoacetic acid, 1×10^{-6} M	15.2	+10
II	Control	12.7	
	Iodoacetic acid, 1×10^{-6} M	14.7	+15
III	Control	12.0	
	Iodoacetic acid, 1×10^{-6} M	13.9	+16
IV	Control	12.0	
	Iodoacetic acid, 1×10^{-6} M	13.8	+15
V	Control	11.0	
	Iodoacetic acid, 1×10^{-6} M	12.8	+16
VI	Control	12.3	
	Iodoacetic acid, 1×10^{-6} M	14.0	+14
VII	Control	12.9	
	Iodoacetic acid, 1×10^{-6} M	14.8	+15

Measurements were made after a 24-hr incubation period at 25° C in basal medium containing 1 % sucrose, 5.7×10^{-6} M IAA, 2×10^{-3} M KCl, 5×10^{-4} M $MnSO_4$ and 0.01 M phosphate buffer, pH 5.0.

* Mean increase for each trial was obtained from 40 sections.

TABLE II

THE EFFECT OF CERTAIN THIOL, DITHIOL AND DISULFIDE COMPOUNDS ON IODOACETIC ACID STIMULATION

ADDITIVES	% DEVIATION FROM CONTROL	EFFECTIVE ACTION
Iodoacetic acid, 1×10^{-6} M	+15	Stimulation
Lipoic acid, 4.9×10^{-6} M	− 2	None
Iodoacetic acid + lipoic acid	+ 1	Reversal
Co A, 1.3×10^{-6} M	− 1	None
Iodoacetic acid + Co A	+16	No reversal
Cystine, 1×10^{-6} M	+ 2	None
Iodoacetic acid + cystine	+13	No reversal
Cysteine, 1×10^{-6} M	+ 4	Small stimulation
Iodoacetic acid + cysteine	+15	No reversal
GSH, 5×10^{-6} M	+ 7	Stimulation
Iodoacetic acid + GSH	+22	Additive effects
GSSG, 5×10^{-6} M	+ 6	Stimulation
Iodoacetic acid + GSSG	+24	Additive effects
BAL, 1×10^{-6} M	− 3	Slight inhibition
Iodoacetic acid + BAL	+13	No reversal
Thioglycollic acid, 1×10^{-4} M	+ 3	Slight stimulation
Iodoacetic acid + thioglycollic acid	+12	Slight reversal
MTA, 4.4×10^{-6} M	− 3	Slight inhibition
Iodoacetic acid + MTA	+11	Slight reversal

Measurements made as in table I.

TABLE III

EFFECT OF LIPOIC ACID ON THE CUMULATIVE ACTION OF IODOACETIC ACID AND REDUCED GLUTATHIONE

ADDITIVE	% DEVIATION FROM CONTROL
Iodoacetic acid, 1×10^{-6} M	+15
GSH, 5×10^{-6} M	+ 6
Iodoacetic acid + GSH	+23
Lipoic acid, 4.9×10^{-6} M	- 1
Iodoacetic acid + GSH + lipoic acid	+ 9

Measurements made as in table I.

thioglycollic acid, GSH and BAL were all without effect upon the stimulation.

Arsenite, a specific reactant for dithio linkages (8), has also been used to bring about enhanced growth of Avena coleoptiles (6). It is often used to inhibit oxidative decarboxylation and causes an increase in the concentration of pyruvate in metabolizing organisms. We obtained a 9% growth increase when arsenite was used at a concentration of 5×10^{-8} M (fig 3). The range of stimulation was short but reproducible. Complete reversal of such growth enhancement was brought about by 4.9×10^{-6} M lipoic acid (table IV). Again, BAL (a dithiol), cysteine, and thioglycollic acid were ineffective.

EFFECT OF INDOLEACETIC ACID ON IODOACETATE STIMULATION: Perhaps the most intriguing finding of the present investigation was the discovery that no stimulation by iodoacetate could be obtained in the presence of the optimal concentration of indoleacetic acid. When 5.0×10^{-5} M growth hormone were used instead of the customary 5.7×10^{-6} M, not only was the stimulation eliminated but all concentrations of iodoacetate were now inhibitory (fig 4). The same concentration of iodoacetate (1×10^{-6} M) which in the presence of 5.7×10^{-6} M indoleacetic acid produced maximum stimulation was now inhibitory to the same extent. The interesting trend toward a lessening of the inhibition at high concentrations was readily reproducible.

Another rather unexpected finding was the observation that 4.9×10^{-6} M lipoic acid which had previously reversed iodoacetate stimulation was now equally effective in reversing the inhibition (table V).

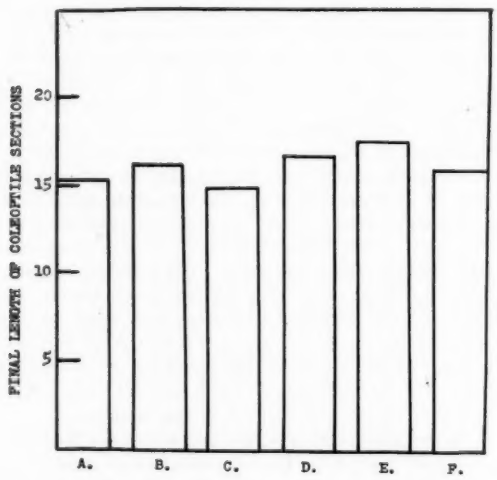

FIG. 2. Effect of CMBA on Avena coleoptile sections and the results of addition of certain sulfur-containing compounds.

A. No additions, control.
B. CMBA.
C. CMBA + lipoic acid.
D. CMBA + thioglycollic acid.
E. CMBA + GSH.
F. CMBA + BAL.

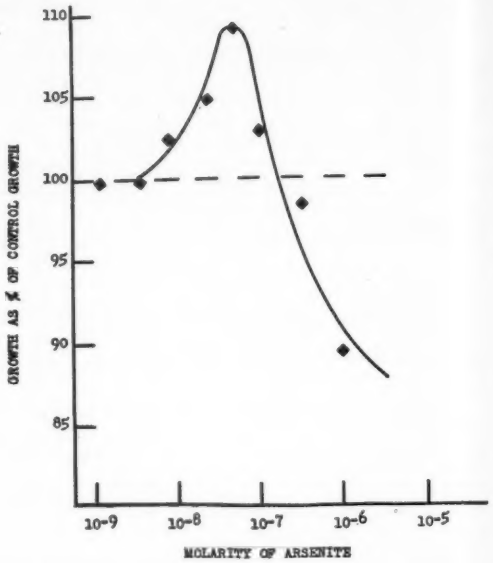

FIG. 3. Effect of low concentrations of arsenite on the elongation of coleoptile sections.

TABLE IV

EFFECT OF CERTAIN SULFUR CONTAINING COMPOUNDS ON ARSENITE STIMULATION OF COLEOPTILE SECTIONS

ADDITIVES	% DEVIATION FROM CONTROL	EFFECTIVE ACTION
Arsenite, 5×10^{-5} M	+ 9	Stimulation
Lipoic acid, 4.9×10^{-6} M	- 2	None
Arsenite + lipoic acid	- 1	Reversal
BAL, 1×10^{-6} M	- 3	None
Arsenite + BAL	+ 7	No reversal
Cysteine, 1×10^{-6} M	+ 4	Small stimulation
Arsenite + cysteine	+11	No reversal
Thioglycollic acid, 1×10^{-6} M	+ 3	None
Arsenite + thioglycollic acid	+ 9	No reversal

Measurements made as in table I.

TABLE V

REVERSAL OF IODOACETIC ACID INHIBITION OF COLEOPTILE SECTIONS

IODOACETIC ACID $\times 10^{-6}$ M	LIPOIC ACID $\times 10^{-6}$ M	% DEVIATION FROM CONTROL
0.01	...	- 4
0.01	4.9	- 1
0.05	...	-10
0.05	4.9	- 2
0.1	...	-13
0.1	4.9	- 1
0.5	...	-19
0.5	4.9	0
1	...	-12
1	4.9	- 2
5	...	- 8
5	4.9	0
10	...	-17
10	4.9	- 4

See table I.

In all cases the inhibition was reversed, and growth was equivalent to the control containing 5.0×10^{-5} M indoleacetic acid.

DISCUSSION

In attempting to explain the foregoing data we should like to suggest the following possible mode of action (fig 5), similar in some respects to that scheme presented by Bonner and Thimann (3). In assigning auxin a role in cell elongation at the position shown,

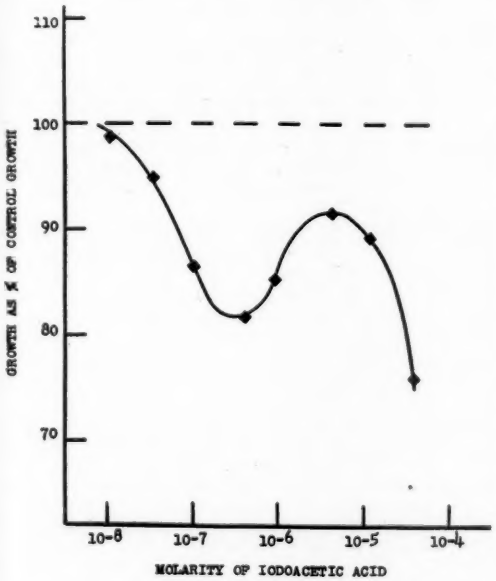

FIG. 4. Effect of iodoacetic acid on coleoptile growth in the presence of 5.0×10^{-6} M IAA.

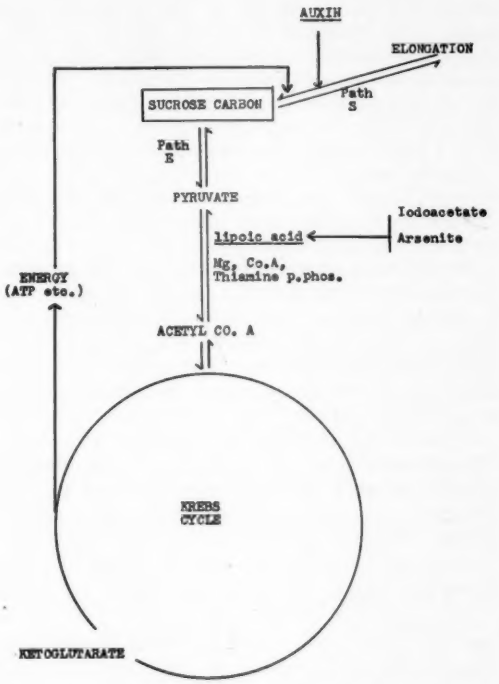

FIG. 5. Scheme for sucrose-carbon distribution. Paths S and E represent carbon utilization for structure and energy respectively.

the assumption is made that sucrose carbon is essential to cell elongation in some unknown capacity and that indoleacetic acid catalyzes the formation of some growth-essential compound from this carbon. Much work has indicated that auxin may enhance the synthesis of cell wall materials from glucose. In addition to its utilization in some unknown manner to satisfy structural requirements for elongation, sucrose must also act as a source of energy for the process. This might be considered a typical Emden-Meyerhof process with most of the energy being supplied through the operation of the Krebs cycle. We believe that iodoacetate may stimulate elongation by readjusting the distribution of sucrose carbon between these two pathways in such a way that the new dynamic equilibrium more nearly satisfies the demand for both structural carbon and energy carbon. To illustrate, if we assume that in the presence of suboptimal concentrations of auxin (i.e., 5.7×10^{-6} M) the process is wasteful of sucrose carbon in that too much is being diverted into the Krebs cycle with the resultant production of excess energy, the removal of lipoic acid by iodoacetate would result in a build-up of three carbon compounds. The new dynamic equilibrium would divert more of the sucrose carbon toward structural requirements and less toward supplying superfluous energy. Higher additions of iodoacetate would finally limit the energy supply to a detrimental degree, re-

sulting first in a lessening of the stimulation and finally in an inhibition of growth. Arsenite, CMBA, and amino-naphthol-sulfonic acid could presumably stimulate in the same manner. The reversal of these stimulations (arsenite and CMBA) by lipoic acid alone indicates either that sulfhydryl groups of lipoic acid have a higher affinity for these agents than do other sulfhydryl compounds as might be expected from the work of Barron and Singer (4) and Stocken and Thompson (8), or that other essential sulfhydryl compounds are present in such excess that a small loss of such non-limiting materials by reaction with iodoacetate has little effect upon the metabolic equilibria.

At the optimal concentration of indoleacetic acid (i.e., 5.0×10^{-5} M) the situation with respect to these additives is quite different. At this point we have an ideal dynamic balance at which the division of substrate carbon between the two processes of energy production and utilization for structure is precisely optimal and finely tuned so that any change must, of necessity, result in decreased growth. Small amounts of iodoacetate now inhibit since they decrease the optimal energy output. Lipoic acid reverses the inhibition by increasing the flow of carbon into the Krebs cycle and hence increasing energy production.

Concentrations of auxin greater than 5.0×10^{-5} M result in a decreased elongation when compared to those grown in the optimal concentration. Presumably, this could be interpreted as indicating too great a utilization of sucrose carbon for structural purposes at the expense of a limited energy supply. This is born out by the fact that concentrations of Co A which were inhibitory at optimal auxin concentration bring about a lessening of the inhibition due to excess auxin (table VI). In a sense, Co A has now become stimulatory. It brings about a new dynamic equilibrium wherein more sucrose carbon flows into the Krebs cycle, resulting in enhanced growth.

TABLE VI

EFFECT OF COENZYME A ON INDOLEACETIC ACID TREATED COLEOPTILE SECTIONS

COENZYME A $\times 10^{-6}$ M	IAA, $\times 10^{-5}$ M		
	5.0	5.7	6.3
	% Deviation from control		
0	*	− 8	−17
0.13 **	− 6	− 5	−13
0.63	− 9	− 1	− 5
1.3	−14	− 7	− 1
2.0	−22	−13	− 7

In addition to indicated conc of coenzyme A, measurements made as in table I.

* Control.

** Preparation in reduced form, 90% pure.

SUMMARY

Stimulation of straight growth of the Avena coleoptile by subinhibitory concentrations of iodoacetate and other sulfhydryl-reactive compounds has been studied in detail.

Such stimulation was prevented by the addition of lipoic acid but not by other sulfhydryl or disulfide-containing compounds.

Stimulation by these compounds was found to be impossible in the presence of the optimal concentration of indoleacetic acid and under such conditions iodoacetate inhibited at all concentrations. This inhibition was prevented by lipoic acid.

It is postulated that such compounds stimulate growth by partially inhibiting the oxidative decarboxylation of pyruvate or α-ketoglutarate. Such an inhibition may result in a more favorable allocation of glucose carbon between two competing, carbon-consuming processes; one utilizing carbon for the production of energy, the other for structurally essential compounds.

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THE DARK FIXATION OF CO_2 BY SUCCULENT LEAVES: METABOLIC CHANGES SUBSEQUENT TO INITIAL FIXATION[1,2]

PAUL SALTMAN, VICTORIA H. LYNCH,[3] GEORGE M. KUNITAKE, CLYDE STITT AND HERBERT SPOLTER

DEPARTMENT OF BIOCHEMISTRY AND NUTRITION, SCHOOL OF MEDICINE, UNIVERSITY OF SOUTHERN CALIFORNIA, LOS ANGELES 7, CALIFORNIA

Succulent plants are characterized by a diurnal rhythm of dark acidification and light deacidification. Concomitant with the acidification there is a loss of stored carbohydrate while in the light, organic acids are converted to carbohydrate (3). That CO_2 fixation in the dark is directly linked with the formation of acids was conclusively demonstrated by Thurlow and Bonner (20) using $C^{14}O_2$ and excised leaves of *Bryophyllum crenatum*. Thomas and his co-workers (19) have confirmed the relationship of CO_2 fixation and acid synthesis by elegant physiological experiments in which CO_2 uptake was correlated with increased acid formation.

Analysis of several compounds synthesized during the dark fixation has been made by Varner and Burrell (22). Excised *B. calycinum* leaves were exposed to $C^{14}O_2$ for 2.5 hours in total darkness, and the organic acids and carbohydrates isolated by chromatography on silica gel columns. The acids isolated were malic, succinic, oxalic, citric, and iso-citric—the same acids which had been identified chemically by Pucher (11). Stutz and Burris (18) extended the work of Varner and Burrell by exposing a wide variety of leaves from higher plants to $C^{14}O_2$ in the dark, separating the acids by column chromatography and measuring their specific activities. Ranson (13) employed the technique of paper chromatography and radioautography to identify aspartate and glutamate as products of 14-hour dark $C^{14}O_2$ fixation of Kalanchoë leaves. Bradbeer (4) has reported the incorporation of $C^{14}O_2$ label into a α-ketoglutarate, pyruvate, and oxaloacetate.

In order to gain further insight into the pathways of $C^{14}O_2$ during the dark metabolism of succulents, the techniques of Calvin, Benson, and co-workers (2) were adapted to this problem. By exposing *B. calycinum* leaves to $C^{14}O_2$ in the dark for varying periods of time and isolating and identifying the products formed, one should be able to elucidate the metabolic interconversions of the organic and amino acids. This paper will describe experiments in which the compounds incorporating radioactivity have been identified and their activities determined during periods of dark fixation from 1 minute to 2 hours. The pattern of compounds isolated suggests the operation of the enzymes of the Krebs tricarboxylic acid cycle as well as the participation of transamination reactions leading to the formation of amino acids from the α-keto acids of the cycle.

METHODS

The methods used in these longer periods of dark fixation of $C^{14}O_2$ are the same as those described in detail in a previous communication (15). A specially constructed apparatus permits excised leaves from *B. calycinum* to be exposed to $C^{14}O_2$ in total darkness. Approximately one gm of young leaves taken from the apex of the plants immediately before use was placed in the apparatus, $C^{14}O_2$ generated from 4 to 5 mg of $BaC^{14}O_3$ (specific activity 0.0282 mc/mg) was admitted, and the reaction terminated at the desired time by homogenization in boiling 80 % ethanol. The extract was filtered, extracted with Skellysolv A (Pentane), and the ethanolic extract concentrated to a volume of 2.0 ml under reduced pressure. Two dimensional chromatography of an aliquot (0.1 to 0.2 ml) of the concentrated extract in 80 % phenol–20 % water (w/w) in the first direction and (79) butanol–(19) acetic acid–(50) water (v/v/v) in the second, was used to separate the compounds. Radio-autographs were made by exposing "no-screen" x-ray film to the chromatogram. Identification of radioactive compounds was made by elution and subsequent cochromatography with known compounds. The activity of each compound was measured directly on the paper with an end window Geiger tube. Derivatives of the α-keto acids were made by disrupting the tissue with 30 ml of 5 *N* H_2SO_4, adding 30 ml of 5 *N* H_2SO_4 saturated with 2,4-dinitrophenyl hydrazine, and forming the hydrazone derivatives as described by Ranson (personal communication). The mixture was allowed to stand for one hour at room temperature and then filtered. The filtrate was extracted three times with 25-ml portions of ethyl acetate. The ethyl acetate was then extracted three times with 25-ml portions of 10 % Na_2CO_3. The Na_2CO_3 solution was acidified to pH 1.0 with cold 5 *N* H_2SO_4 and the hydrazones extracted into ethyl acetate. The ethyl acetate fraction was dried over anhydrous Na_2SO_4 and then concentrated to 2.0 ml under reduced pressure. The hydrazones were separated by paper chromatography by the method of Isherwood and Cruickshank (9), and were eluted and identified by cochromatography with authentic derivatives of the keto-acids.

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[2] This investigation was supported in part by a research grant from the National Science Foundation, and in part by the Tobacco Industry Research Committee. The facilities of the Allan Hancock Foundation were generously provided.

[3] Present address: Carnegie Institution of Washington, Stanford, California.

Results and Discussion

A radio-autograph for a 1-hour dark fixation is presented in figure 1. The compounds identified conclusively are: malate, citrate, isocitrate, succinate, fumarate, aspartate, asparagine, glutamate, glutamine, alanine, glycine, and serine. The activities of these compounds expressed as percent of total activity fixed at the time intervals tested are presented in table I. After 60 minutes no new labeled compounds appear, although total activity in these compounds continues to increase. It should be noted that neither phosphorylated intermediates of glycolysis nor carbohydrates appear during dark CO_2 fixation. Table II presents the activities of the various α-keto acids isolated as the hydrazone derivatives.

Probable metabolic pathways by which the labeled compounds may be formed after the initial fixation of $C^{14}O_2$ as oxaloacetate are suggested in figure 2.

The classical Krebs tricarboxylic cycle would appear to play a key role in succulent metabolism. The preparation of biochemically active mitochondrial particles from green pea leaves by Smillie (16) indicates the functioning of this metabolic system in leaf tissue. The previous inability of many workers (5) to isolate such preparations seems entirely due to procedural difficulties and not to the absence of mitochondria in green tissue. Stutz and Burris (18) found that the specific activities of some of the acids of the cycle failed to reach equilibrium in *B. calycinum* leaves exposed to $C^{14}O_2$. They interpreted their results to indicate that the cycle, if operative at all, must be quite sluggish. However, they fail to consider the rates at which some of the acids, particularly malic, citric, and iso-citric are removed from the metabolic activities of the cycle into the vacuole or into some other more static area of the cell. Evidence from our laboratory, to be presented elsewhere, indicates that all organic and amino acids are not equally available for metabolism. The experiments of Tolbert

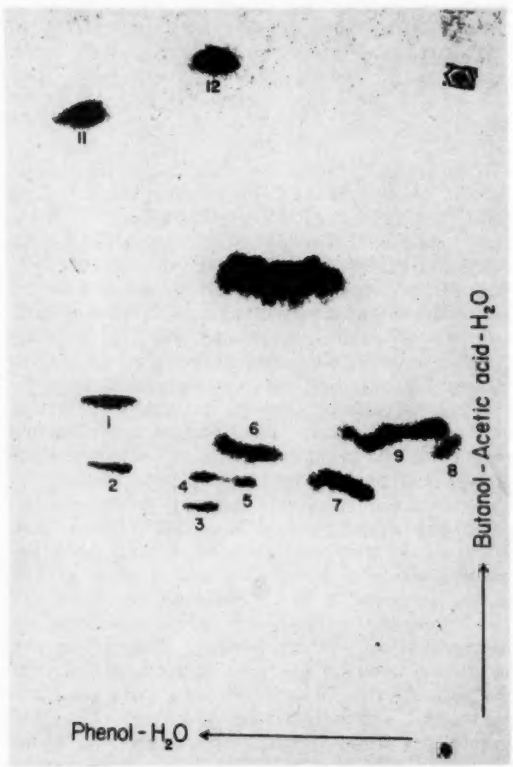

Fig. 1. Photographic reproduction of radioautogram from two dimensional paper chromatogram of extract from 60-min dark fixation of $C^{14}O_2$ by *Bryophyllum calycinum*. Compounds identified are: 1. alanine; 2. glutamine; 3. asparagine; 4. glycine; 5. serine; 6. glutamate; 7. aspartate; 8. citrate; 9. isocitrate; 10. malate 11. fumarate; 12. succinate.

Table I

Products from the Dark Fixation of $C^{14}O_2$ by Bryophyllum calycinum Leaves *

Compound	1 Min	5 Min	10 Min	30 Min	60 Min	120 Min
Malate	60	65	57	71	61	75
Aspartate	20	10	8.7	7.6	11	4.1
Isocitrate	11	10	14	7.5	15	8.2
Citrate	4.1	4.3	3.4	4.6	6.1	5.3
Glutamate	4.0	2.1	3.9	2.6	2.1	2.0
Glutamine	0.3	0.5	3.8	0.7	0.2	0.5
Alanine	0.0	1.3	3.4	0.4	0.2	0.3
Succinate	0.0	1.9	1.0	0.5	0.9	0.7
Fumarate	0.0	0.8	1.8	0.7	0.4	0.5
Serine	0.0	0.0	0.0	0.2	0.3	0.1
Glycine	0.0	0.0	0.0	0.0	0.2	0.2
Asparagine	0.0	0.0	0.0	0.0	0.3	0.1
Totals	99.4	95.9	97.0	95.8	97.7	97.0

* All values as percentage of total C^{14} counted on the chromatogram. Small amounts of unidentified compounds and the origin account for the percentages not listed.

These are the results of a single experimental run, but are typical of 3 expts.

Table II

Activities of 2,4-Dinitrophenylhydrazone Derivatives after a 10-Minute Dark Fixation of $C^{14}O_2$ by Bryophyllum calycinum Leaves *

Keto acid	% Activity
Oxaloacetate	38
α-Ketoglutarate	28
Pyruvate	33
Total	99

* Values are given as percentage of total C^{14} counted on the chromatogram. Small amounts of unidentified hydrazone derivatives and the origin account for the percentages not listed.

These are the results of a single experimental run, but are typical of 4 expts.

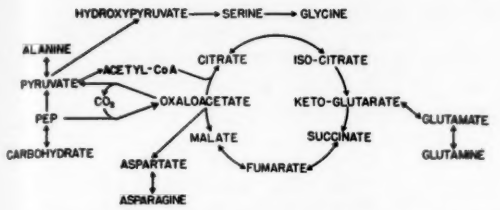

FIG. 2. Proposed metabolic pathways involved in the dark fixation of CO_2 by *Bryophyllum calycinum* leaves. The pathway for the biosynthesis of serine from pyruvate via hydroxypyruvate is hypothetical.

(21) with large uni-cellular algae also indicate that differential transport of organic compounds is involved in plant metabolism. Ramstad and Lieberman (12) have expressed the view that the Krebs cycle is not functioning in succulent metabolism. Their fundamental assumptions with respect to the concentrations of acids and the equilibrium of citrate, iso-citrate, and cis-aconitate could be explained by differential transport mechanisms.

Further evidence supporting an active cycle in succulents is found in the experiments of Thomas (1[illegible] who demonstrated that dark CO_2 fixation is markedly inhibited at low oxygen levels. Not only is the cycle sensitively dependent upon oxygen for optimal activity, but also, anaerobiosis increases the amount of adenylate acceptor (i.e., ADP[4]) available. Since PEP can either be carboxylated to yield oxaloacetate, or transfer its phosphate group to ADP to yield ATP, anaerobiosis would favor the later reaction, and, as a direct consequence, a decrease in the carboxylation reaction would be observed. It is difficult to conceive of metabolic sequences other than the Krebs cycle that would account for the pattern of compounds identified during the dark fixation of CO_2 by succulent leaves.

The rapid formation of glutamate could occur from α-ketoglutarate by either transamination or by the direct amination through the action of glutamic acid dehydrogenase. The activity found in alanine and aspartate would appear to be dependent upon synthesis via the activity of the transaminases (26). That the oxaloacetate must "shuttle" via malate to the symmetrical molecule of fumarate and back to oxaloacetate is apparent from the activity found in alanine. Were this "shuttle" not operative, the decarboxylation of oxaloacetate would yield non-radioactive pyruvate and consequently, inactive alanine. The presence of α-ketoglutarate, oxaloacetate, and pyruvate in plant tissue has been demonstrated by Virtanen (23) and by Barker and Mapson (1). We have confirmed and extended their observations by isolating 2,4-dinitrophenylhydrazone derivatives of these keto acids which contained radioactivity after a 10-minute exposure to $C^{14}O_2$ in the dark. Thus the key α-keto acids of the Krebs cycle seem to be directly involved in the dark metabolism of the succulents. That glutamine incorporates label coincidentally with the glutamate is evidence for a very active glutamine synthetase in Bryophyllum leaves similar to the enzyme system studied by Elliot (7) and Webster (24) in a variety of plant tissues. The asparagine synthease (25) enzyme is much slower than the glutamine enzyme as demonstrated by the relatively late appearance of radioactive asparagine despite the fact that aspartate is one of the first compounds to incorporate label.

The proposed pathways for the synthesis of serine and glycine are based only on the observation that serine incorporates radioactivity before glycine. Much evidence is now available to indicate that serine is the precursor to glycine in animal tissue. Sallach (14) has administered carboxyl-C^{14} glyceric acid to rats and has observed its direct conversion to carboxyl labeled serine. He has also shown the presence of an active transaminase which transfers the amino group of alanine to hydroxypyruvate. Kitt (10) has incubated glucose-1-C^{14} with tumor tissue for short periods of time and has demonstrated that serine is labeled prior to glycine. To explain these results, Kitt postulates a pathway leading from phosphoglycerate to phosphohydroxypyruvate to phosphoserine. Confirmation for this metabolic scheme for serine biosynthesis is given by the study by Greenberg and Ichihara (8) who with the use of preparations from rat liver, were able to isolate and identify the phosphorylated intermediates in the metabolic pathway. Stafford et al (17) have investigated an enzyme from parsley which catalyzes the reaction

$$\text{DPNH} + \text{H}^+ + \text{hydroxypyruvate} \rightleftharpoons \text{D-glycerate} + \text{DPN}^+.$$

Once labeled carbon has been incorporated into pyruvate or phosphopyruvate, as is indicated by our experiments, a reversal of glycolysis would lead to phosphoglycerate. We are not able at this time to ascertain whether a phosphorylated or non-phosphorylated pathway is operative in the synthesis of serine by higher plants.

We have been unable to demonstrate the incorporation of label into oxalate by cochromatography of our extracts with carrier oxalate. This finding is in contrast to the studies of Varner and Burrell (22) and Stutz and Burris (18) who have isolated the oxalate by column chromatography and have shown appreciable incorporation of $C^{14}O_2$. There was no detectable activity associated with lactate, glyoxylate, and glycolate, compounds which are associated with the organic acid metabolism of higher plants (6).

SUMMARY

1. Compounds which incorporate $C^{14}O_2$ during dark fixation by *Bryophyllum calycinum* leaves have been identified by two dimensional paper chromatog-

[4] The following abbreviations are employed: PEP, phosphoenolpyruvate; DPN^+, oxidized diphosphopyridine nucleotide; DPNH, reduced diphosphopyridine nucleotide; ATP, adenosine triphosphate; ADP, adenosine diphosphate.

raphy. The compounds detected are: malate, citrate, iso-citrate, succinate, fumarate, aspartate, asparagine, glutamate, glutamine, alanine, glycine, and serine.

2. The kinetics of the incorporation has been studied by exposing the excised leaves to $C^{14}O_2$ for periods of time from one minute to 2 hours.

3. The pattern of incorporation observed in the organic and amino acids suggests the operation of the Krebs tricarboxylic acid cycle in its entirety. Amino acids are synthesized from their respective α-keto acid analogues by transamination or by reductive amination.

4. Labelling is observed in serine before it can be detected in glycine during the dark fixation of $C^{14}O_2$ by succulents.

We wish to thank Drs. J. Bonner and G. Laties of the California Institute of Technology for their valuable advice and suggestions as well as for the plants used in these experiments.

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THE PRODUCTS OF CO_2 DARK FIXATION IN LEAVES OF LONG- AND SHORT-DAY TREATED KALANCHOË BLOSSFELDIANA[1,2]

GEORGE M. KUNITAKE, PAUL SALTMAN AND ANTON LANG

DEPARTMENT OF BIOCHEMISTRY AND NUTRITION, UNIVERSITY OF SOUTHERN CALIFORNIA, LOS ANGELES 7, AND DEPARTMENT OF BOTANY, UNIVERSITY OF CALIFORNIA, LOS ANGELES 24, CALIFORNIA

As shown by Gregory, Spear and Thimann (2), photoperiodic induction results in a striking increase in the rate of dark fixation of carbon dioxide by the short-day plant *Kalanchoë blossfeldiana*. The "extra" CO_2 thus fixed in the dark period of a short day is released in the following light period, and the normal pattern of CO_2 exchange in green plants may appear reversed, CO_2 being absorbed in the dark and evolved in the light. Brief light interruptions in the middle of the dark periods prevent the development of the short-day pattern of CO_2 fixation. Since such "light flashes" suppress also the flower-inducing action of short days, and because of some additional parallels between increased CO_2 dark fixation and flower induction it seems attractive to assume a causal relation between the two processes.

The present experiments were undertaken in order to discover whether the difference in the dark fixation of carbon dioxide by photoperiodically induced and noninduced Kalanchoë is qualitative or quantitative in nature. This was done by exposing leaves from induced (short-day treated) and non-induced (long-day grown) plants to radioactive CO_2 and determining by means of paper chromatography the compounds in which the label appeared.

MATERIAL AND METHODS

The work was performed with the variety Tom Thumb of *Kalanchoë blossfeldiana*. The original plants, grown from seed, were kindly supplied by Dr. A. M. Kofranek, Department of Floriculture, U. C. L. A.; later, they were propagated by cuttings. The plants were grown with a single shoot, all side shoots being removed as soon as they appeared. Long-day treated plants received at least 19 hours of light daily (supplementary light of 30 ± 5 ft-c from 5 P.M. to 2 A.M.) and remained in a strictly vegetative condition. Short-day treatment consisted of 9-hour light periods (8 A.M. to 5 P.M.) and 15-hour dark periods. Long- and short-days were given in the greenhouse, and while no attempt was made at controlling day temperatures, night temperatures were maintained between 18 and 20° C, occasionally rising to 25° C. Under these conditions, and depending on their age, the plants require a minimum of 6 to 10 short days for a macroscopic flowering response, and optimum response is obtained with about 20 short days.

The exposure to radioactive CO_2 was carried out in the 11th to 12th hour of the dark period, since according to the graphs in Gregory et al (2) CO_2 fixation in long dark periods usually reaches its maximum rate at that time. The noninduced individuals were thus in their first long dark period, in which CO_2 fixation is not yet appreciably different from fixation in the short dark periods of long days. The number of short days which the induced plants had been given will be indicated below.

CO_2 exposure and analysis of the fixation products followed the methods developed by Saltman et al in their studies on metabolism of succulent leaves (9). In each experiment, one plant from each condition (noninduced, and induced for different periods of time) was used. Two opposite leaves which had reached at least three quarters of their final length were selected, care being taken to have closely comparable leaves from induced and noninduced plants. The leaves were detached and rapidly weighed in dim green safety light which had been found to be photoperiodically ineffective ("natural green" incandescent lamps, 15 w., indirect illumination). They were then transferred into the incubation chamber and exposed to an atmosphere containing $C^{14}O_2$ generated from $BaCO_3$ with C^{14} (specific activity, 0.06 mc/mg). The CO_2 content of the atmosphere was about 1 %.

After suitable exposure intervals the leaves were killed, homogenized and extracted with 80 % ethanol. The extract was filtered and concentrated in vacuo. An aliquot was assayed for total radioactivity with an end-window Geiger-Mueller tube. Another aliquot was subjected to two-dimensional ascending chromatography on Whatman No. 1 filter paper. The solvent systems used were phenol (80): water (20), (w/w), and n-butanol (79) : acetic acid (19) : water (50), (v/v/v). Radioautograms of the chromatograms were made with "no-screen" x-ray film.

In most experiments the leaves were killed with boiling 80 % ethanol, homogenizing and extraction were performed in the incubation chamber, and the subsequent steps were carried out in the laboratory at room temperature. However, because of the possibility that a highly labile compound labeled with C^{14} might be destroyed or lost under these conditions, one experiment was done using additional precautions. The killing was done with liquid nitrogen and all subsequent manipulations including chromatography and radioautography were performed in the cold and in the dark (or in green safety light where vision was required). The results obtained by this procedure were, however, identical with those of the other experiments.

RESULTS

In all, three separate experiments were performed using both 10- and 30-minute dark exposure periods. The results of a representative experiment are sum-

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TABLE I

TOTAL ACTIVITY FIXED BY LEAVES OF INDUCED AND NONINDUCED KALANCHOË BLOSSFELDIANA AFTER 10 MINUTES EXPOSURE TO $C^{14}O_2$ IN DARKNESS

SHORT DAY	MG $BaC^{14}O_3$	GM LEAVES	CPM	
			TOTAL	CORRECTED *
1st	6.6	1.10	48,300	6,650
23rd **	4.1	1.31	440,000	82,500
32nd ***	4.7	1.60	151,500	20,100

* Cpm/gm leaves × mg $BaCO_3$.
** Plant without visible flower buds.
*** Plant with visible flower buds.

marized in tables I and II. The exposure time to $C^{14}O_2$ in this experiment was 10 minutes. Experiments with 30 minutes exposure time gave similar results. Despite minor variations in the percent activity in the various compounds, the distribution of label was essentially the same.

The data for the total activity fixed by leaves from induced and noninduced plants, and extractable with 80 % ethanol, are presented in table I. It is clear that the induced leaves have a greatly enhanced ability to fix CO_2. Upon the appearance of visible flower buds this ability seems to be markedly lowered. However, this may be a secondary effect. The production of flower buds markedly increases the rate of ageing of the leaves of Kalanchoë, and leaves from flower-bearing and flowerless individuals may not be comparable physiologically, even though they are of the same absolute age (size).

The compounds which incorporate radioactivity in both the induced and noninduced leaves are listed in table II. All labeled compounds found in the induced plants were found in the noninduced. No difference in the patterns of labeled compounds could be detected. It is interesting to note that the labeled compounds found in *K. blossfeldiana* are the same as those found in *Bryophyllum calycinum* (10).

The percent of total activity present on the chromatograms found in the individual compounds is also shown in table II. Again, there seems to be no significant difference between induced and noninduced leaves; the relative amounts of each compound are the same. It is therefore probable that the entire increase of radioactivity in the induced leaves can be accounted for by the increase of radioactivity in the products of noninduced fixation.

CONCLUSIONS

The results described above show that short-day treatment results in a greatly increased capacity for dark fixation of carbon dioxide in the leaf of Kalanchoë, thus confirming the work of Gregory et al (2) with a different technique, and that the CO_2 fixed by induced leaves is incorporated into the same compounds as in leaves from long-day grown plants. It might be argued that in our procedures we lost a "new," highly unstable product of CO_2 dark fixation which is present in leaves of induced plants only. However, such an assumption is improbable for a number of reasons. Firstly, added precautions in handling the material after exposure to $C^{14}O_2$, excluding any action of light and elevated temperature (see under Material and Methods), had no effect on the results. Secondly, the relative increase of radioactivity in the individual products of $C^{14}O_2$ fixation corresponds very closely to that in total $C^{14}O_2$ fixation, suggesting, as emphasized before, that the total increase of CO_2 fixation by induced leaves is entirely based on the increase in the individual products of the noninduced fixation. Thirdly, Neyland and Thimann (6) have recently shown that the increase of CO_2 dark fixation in short-day treated Kalanchoë can be quantitatively accounted for by the increase in the organic acid fraction of the plants. This evidence, considered in its entirety, suggests that short-day treatment affects CO_2 fixation in Kalanchoë in a strictly quantitative manner. It does not create a new, additional pathway for CO_2 fixation, nor does it change the relative rates of CO_2 transfer between different compounds (rates of decarboxylation and carboxylation). It results, however, in an increase of the capacity for the initial incorporation of CO_2 and/or its transfer to subsequent compounds. If any one of the enzymes which participate in these reactions, operates at the limit of its capacity, an increase of the latter by short-day treatment of the plant would account for the observed changes in CO_2 dark fixation in induced Kalanchoë plants.

If increased dark fixation of CO_2 under short-day conditions and photoperiodic induction of flower formation are causally related, the relation would appear to be a rather nonspecific one. One can, for example, assume that the rise in the level of the products of CO_2 dark fixation, or some of these products, is a premise for the activity of some of the biochemical processes leading to flower initiation. It is also conceivable that the increase of acidity in the leaf cells, which will probably result from the increased organic acid content, is important in relating to flowering. However, Garner et al (1) did not find an increase, but rather a decrease in acidity of the expressed sap

TABLE II

PRODUCTS FROM THE 30-MINUTE DARK FIXATION OF $C^{14}O_2$ BY INDUCED AND NONINDUCED KALANCHOË BLOSSFELDIANA LEAVES

COMPOUND	NONINDUCED	INDUCED
Malate	61.9	73.0
Aspartate	8.3	4.7
Glutamate	4.2	2.4
Alanine	2.6	2.0
Glutamine	0.6	0.5
Citrate, isocitrate	13.4	15.3
Succinate fumarate	1.0	1.0

All values are percentage of total C^{14} counted on the chromatogram. Small amounts of unidentified compounds and the origin account for percentages not listed.

from the apical region of Cosmos (a nonsucculent short-day plant) upon photoinduction. But our results do not indicate that some product of short-day CO_2 dark fixation has a specific and direct significance in photoperiodic induction.

It ought also to be borne in mind that the causal relation of CO_2 dark fixation and flower induction, while suggestive, cannot be considered as unequivocally established. In the experiments of Gregory et al (2), the most spectacular increase of CO_2 uptake in the dark periods occurs after the 15th short day, that is after photoperiodic induction of flower formation has been consummated. Increased CO_2 fixation thus may be associated with the "induced state" (or even with the early processes of actual flower formation) rather than with the "act of induction" (see 8). Langston and Leopold (5) have reported that exclusion of CO_2 from the atmosphere during the dark periods of photoinductive cycles results in a reduction of the number of flowers or inflorescences produced by plants of Biloxi soybean and Xanthium, two short-day species. It is, however, dubious whether the total flower or inflorescence number per plant is a satisfactory criterion for the effectiveness of photoperiodic induction. Photoinduction is usually measured by the number of flower-bearing nodes in Soja (3), and by the early "stages" of inflorescence development in Xanthium (4, 7). There is a simple and highly reproducible relation between these criteria and the amount of induction. No such information is available for the relation between photoinduction and the total number of flowers formed on the plant.

Summary

Dark fixation of carbon dioxide by leaves of induced and noninduced plants of *Kalanchoë blossfeldiana* was studied by means of 10- to 30-minute exposures to $C^{14}O_2$. Short-day treatment resulted in an increase of the rate of dark fixation. However, neither the pattern of labeled compounds, nor the relative distribution of radioactivity in these compounds showed any significant differences between leaves of induced and noninduced plants. It is concluded that the increase in induced CO_2 dark fixation is based on the increase in the products of noninduced fixation, and that the effect of short days on CO_2 dark fixation in Kalanchoë is a quantitative one. The relation of CO_2 dark fixation and photoperiodic induction of flower formation is briefly discussed.

Since the preparation of this manuscript the article by Sen, S. P. and Leopold, A. C. (Plant Physiol. 31: 323–329. 1956) appeared. Their conclusions with respect to the causal relationship between dark CO_2 fixation and flowering do not differ fundamentally from our own. However, the pattern of labeling observed by them for their plants (soy bean, Xanthium and barley) are quite different from our own. Further investigation is needed to explain this difference.

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STUDIES ON THE ORGAN OF PRODUCTION OF THE NATURAL GIBBERELLIN FACTOR IN HIGHER PLANTS[1,2]

JAMES A. LOCKHART

DIVISION OF BIOLOGY, CALIFORNIA INSTITUTE OF TECHNOLOGY, PASADENA, CALIFORNIA

The gibberellins have come to be recognized as of major importance in the physiology of higher plants. The application of gibberellin has been shown to change the phenotype of many dwarf plants to that of the normal (1, 6). It has been found to replace the low temperature and long day requirements for flowering of a biennial plant (2), and to reverse the light inhibition of stem growth in dwarf and normal peas (3) and Perilla (4). Extracts containing gibberellin activity, as measured by the specific response of single-gene, dwarf mutants, have been made from the seed of several higher plants (9); providing final proof for the existence of gibberellin-like compounds in higher plants, and making virtually certain the assumption that the physiological responses indicated above are controlled in higher plants by native gibberellin-like compounds.

One of the important physiological questions which arises in further investigations of the physiology of the gibberellins is the location of the region of production of the natural gibberellin factor in the plant. Specialization in plants is not so great as it is in the higher animals; nevertheless, the production of hormones has been found to be restricted to specific tissues in the higher plants. The leaves, roots, stem tip, or in seedlings, the cotyledons, have been shown to produce one or more hormones which act in the correlation and regulation of growth (cf 7).

The present paper will present evidence indicating that the natural gibberellin factor, necessary for maximum stem elongation in normal pea seedlings, is produced in the stem tip.

MATERIALS AND METHODS

Alaska pea seed (Ferry-Morse Seed Co., Los Angeles) a non-dwarf variety of *Pisum sativum*, were washed, soaked for 5 to 6 hours in water, and placed in moist vermiculite in stainless steel trays. They were grown at 25° C in complete darkness, except for brief exposures to weak blue light during watering and experimental manipulations. The plants were used when they were 5 to 6 days old; when the second internode was 2 to 3 cm long, or a day earlier, as the second internode was just beginning elongation, as indicated in the individual experiments.

The dwarf pea used here was Morse's Progress #9, obtained from Ferry-Morse Seed Company, Los Angeles. It has been shown to respond to applied gibberellin (3).

The gibberellin used in these experiments consisted of mixtures of gibberellin A and gibberellic acid.[3] It was usually applied as a 0.004-ml ethanol drop to the apex, at a concentration giving a dose of 1 μgm per plant. Lanolin solutions of growth substances were made up by melting a weighed amount of lanolin and adding indoleacetic acid (IAA) or gibberellin dissolved in a small amount of ethanol. That significant destruction of the IAA did not occur was demonstrated by the formation of large swellings below the cut tip of the seedlings when the higher concentrations of IAA were used.

The grafts were made by the method of Paton and Barber (5). The stocks were cut off at the vertex of the hook and the stem was slit radially downward 2 to 3 mm. The scion, which included 2 to 3 mm of stem below the hook, trimmed wedge-shaped, was inserted into the slit stock and held in place with a small rubber band.

EXPERIMENTAL RESULTS

One method of establishing the organ of production of a growth hormone, if the hormone has first been isolated, is to remove from the plant the organ suspected as the source; and then attempt to reproduce the response normally elicited by this organ by application of the pure hormone. If the added substance can, to a large extent, replace the organ in restoring normal growth, then the presumption is strong that the organ removed normally supplies to the plant a comparable substance. It may be noted that the converse conclusion is not valid.

Figure 1 illustrates the results of a typical experiment, in which various possible sites of gibberellin synthesis were excised from the seedlings. Five-day-old dark-grown Alaska pea seedlings, in which the second internode was just beginning to elongate, were removed from the vermiculite and either the roots, the cotyledons, or both were excised. The seedlings were then placed in glass bottles containing Knop's nutrient solution and 1 % sucrose. To a portion of each group gibberellin was added to the stem tip as an alcoholic drop. The growth of the second internode in darkness was measured 24 and 48 hours later. Removal of the cotyledons (but not the roots) results in a marked inhibition of stem growth within 24 hours. Gibberellin does not affect the growth of these plants any more than it affects the controls.

Over a 48-hour period an effect of removal of the roots on stem growth becomes evident. Figure 2 illustrates the results of two separate experiments, car-

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[2] The work reported here was supported in part by the Herman Frasch Foundation.

[3] Samples obtained by Prof. James Bonner from Dr. F. H. Stodola, U.S.D.A., Peoria, Illinois and Dr. Reed Gray, Merck and Co., Inc., Rahway, New Jersey.

ried out as described above, and measured after 48 hours. In this case an influence of both roots and cotyledons is apparent, and it is clear that gibberellin does not reverse the inhibition of growth in either case. These results fail to provide evidence that the natural gibberellin factor is produced in either the cotyledons or roots. As indicated above, these results fail to provide evidence for or against the production of gibberellin in these organs.

A further possibility would be that the natural gibberellin factor is produced in the tip of the plant and is translocated downward in the stem. To test this possibility the tips of 5- to 6-day-old pea seedlings were cut off at the vertex of the hook, and lanolin, to which in some cases IAA had been added, was applied to the cut surface. Since auxin is known to be produced in the tips of seedlings, the effect of added IAA on the rate of elongation of the stem was investigated at the same time.

In order to eliminate any effects of auxin on cell division at the tip, and measure more precisely the rate of growth after treatment, as well as make possible direct comparison with intact seedlings, two marks were made on the stem with lanolin-carbon ink. The upper mark was placed 2 mm below the tip and the second mark 5 mm below the first. Growth was measured as the elongation of the marked region during the experimental period.

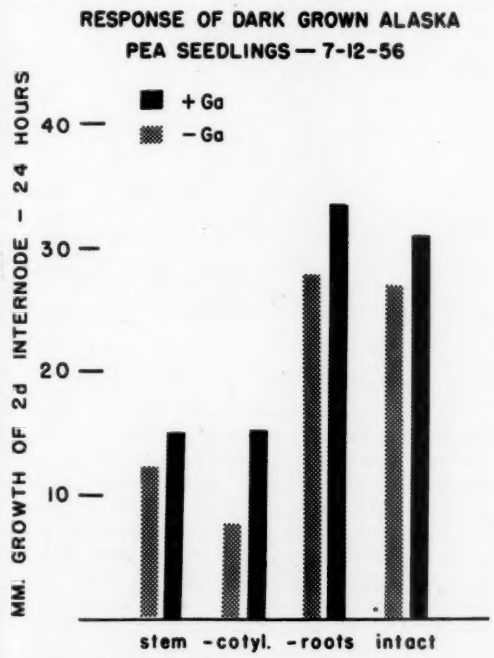


FIG. 1. Growth of 5-day-old Alaska pea seedlings in 24 hours in response to removal of the roots (−roots), the cotyledons (−cotyl.), or both (stem) compared to the intact control. The effect of gibberellin (1 μgm/plant) is shown by the darker bars.

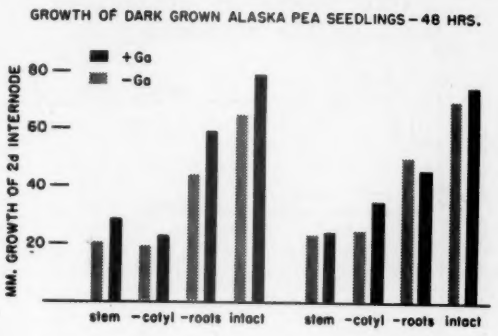


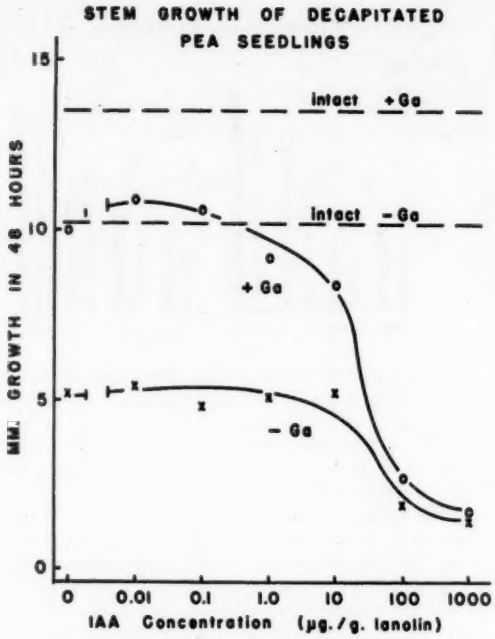
FIG. 2. Growth of 5-day-old Alaska pea seedlings in 48 hours. Treatments identical to figure 1.

Figure 3 illustrates the effects of various concentrations of IAA on the elongation of decapitated pea stems, in the presence or absence of applied gibberellin. The extent of elongation is compared with the elongation of intact plants. The data illustrated represent the average responses of 7 separate experiments. It may be seen that gibberellin but not IAA restores the growth of the decapitated plant virtually to that of the intact controls. While the average data indicate a significant response of the intact plants to gibberellin, it may be noted that a maximum gibberellin response of decapitated plants has been observed in experiments in which the intact plants showed no response to gibberellin.

The results of a typical experiment in which the time course of growth was measured are presented in figure 4. The marked 5-mm section of intact plants elongates in 24 hours from an initial length of 5 mm to a final length approaching 20 mm. The marked section of those plants which were decapitated and to which lanolin alone was applied reached a final length of only 8 to 9 mm, illustrating the effect of decapitation. Application of 1 μgm gibberellin to the decapitated plants almost completely restored the growth capacity of these stems. A typical response to IAA (0.01 μgm/gm lanolin) is also shown. While IAA is capable of promoting growth to some extent, it has not been found, in these experiments, to cause a response comparable to that caused by gibberellin.

It appears clear, then, that gibberellin but not IAA may almost completely replace the stem tip (the apical bud) in promoting the elongation of the growing region of the pea stem. The effective replacement of the tip by gibberellin represents strong evidence that the natural gibberellin factor is produced in the tip of the pea seedling. Nevertheless, independent evidence for this conclusion would be very desirable.

Attempts were therefore made to graft stem tips of the normal Alaska pea onto dwarf stems to determine whether the normal tip would induce greater growth in the elongating region of the dwarf stem, as it should if the tip is producing and exporting the natural gibberellin. Although many of the grafts



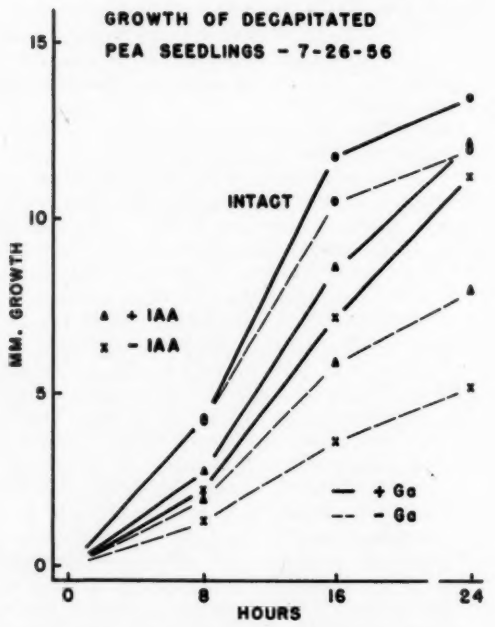


FIG. 3 (*above*). Growth of the subapical 5 mm of the stem of Alaska pea seedlings in response to IAA and gibberellin (1 µgm/plant). Average of 7 separate experiments.

FIG. 4 (*below*). Time course of growth of a typical experiment as described above. IAA concentration is 0.01 µgm/gm lanolin.

were successful, no stem elongation could be observed in the decapitated and grafted dwarf plants. The success of the grafts and the healthy state of the scions is indicated by the growth of the normal tip, after the usual delay of several days. This time lag before graft union occurs suggested that the gibberellin from the tip, even if present, might not pass the graft, at least until tissue union was effected. Such, apparently, is the case with the movement of caulocaline from the base to the tip of the pea (8). To test this possibility, Alaska tips were grafted onto Alaska stems, and the growth of the elongating region of the stock was observed. Although the scions remained healthy and eventually resumed growth, no growth was observed in the stock. Thus the factor from the tip necessary for elongation of the subjacent stem does not reach the stock, at least until tissue union has taken place. Separate experiments indicated that after a delay of as little as 24 hours the decapitated stems will no longer respond to an application of gibberellic acid. Auxin can hardly be limiting growth in this case since it has been shown above that auxin is not limiting the rate of elongation of the stem of decapitated plants.

Several reasons may be suggested to explain the lack of promotion of growth below the graft. Although the tips were cut to include 2 to 3 mm of stem below the hook, and the tips later resumed normal growth, it is possible that the injury of cutting may temporarily stop the production of gibberellin. It may also be suggested that a precursor must move up in the vascular system from the base. Further possibilities may be that gibberellin moves best through the vascular system, or that it is inactivated at the cut surfaces. Studies on the movement and metabolism of gibberellins may best be left for other reports.

The results of the grafting experiments appear to be consistent with the hypothesis that a natural gibberellin is produced in the tip of the pea seedling and is required for maximum elongation of the subjacent stem. The natural gibberellin factor may not cross a graft juncture, at least until tissue union has been effected.

CONCLUSIONS AND SUMMARY

It has been demonstrated that applied gibberellin can replace almost completely the stem tip in promoting the elongation of the subapical region of the pea stem. This effect of the gibberellin cannot be replaced by IAA. It is concluded that the natural gibberellin factor of the pea plant is produced in the stem tip. Gibberellin cannot replace either the roots or cotyledons in the promotion of stem growth. Grafting experiments indicate that the natural gibberellin does not reach the stock across a graft, at least until after tissue union has occurred.

The author wishes to acknowledge the technical assistance of Miss Marjorie Armstrong, Mrs. Mary Lou Whaling and Mr. William Purves with many of the experiments reported here.

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MECHANICAL PROPERTIES OF THE AVENA COLEOPTILE AS RELATED TO AUXIN AND TO IONIC INTERACTIONS[1,2]

TAKASHI TAGAWA[3] AND JAMES BONNER

Kerckhoff Laboratories of Biology, California Institute of Technology, Pasadena, California

The present paper is concerned with the elasticity and plasticity of the Avena coleoptile. This is of interest because it is known that the extension rate of such coleoptiles is determined by the rate at which its cell walls stretch under the load imposed by the turgor pressure of the cell contents. The growth substance, auxin, increases extension rate of plant tissue. The osmotic concentration of Avena coleoptile tissue is not directly influenced by auxin application (5). That the growth promoting effect of auxin is associated rather with an increase in the plasticity and to some extent in the elasticity of the cell wall was first suggested by experiments of Heyn (3, 4) and by Söding (6, 7). Heyn measured deformation of tissue under load and found that the plastic component of such deformation is increased by pretreatment of the tissue with auxin.

If we are to know how auxin increases cell wall plasticity it will be of evident interest to discover first what materials or forces contribute to the rigidity of the wall. It will be shown in this paper that the physical properties of the Avena coleoptile are dependent upon the nature of the inorganic ions exchangeably bound by the tissue. These ions are held by nonliving as well as by living tissue, possibly by pectic materials of the wall structure. This suggests that pectic materials may play an important part in determination of the plasticity of the cell walls of the Avena coleoptile.

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[3] Professor of plant physiology, Hokkaido University, Sapporo, Japan. This work was conducted during the tenure of a Japanese Government Fellowship at the California Institute of Technology.

Materials and Methods

Avena seedlings (var. Siegeshafer) were used throughout the present investigation. The seeds were sown in stainless steel trays containing vermiculite previously moistened with distilled water. They were allowed to germinate and grow in a standard Avena room at 25° C and a relative humidity of 90 %. Additional water was sprinkled over the seedlings approximately 24 and 12 hours prior to harvesting. They were harvested when 96 hours old. At this time a selection was made from those plants possessing coleoptiles 33 ± 1 mm in length. The selection of uniform coleoptiles contributes an important element in obtaining both precise and reproducible results.

From each selected coleoptile one 20-mm section was cut 3 mm from the tip of the coleoptile. Primary leaves were removed from all sections. The sections were floated on the surface of the test solutions for incubation periods ranging from 0 to 120 minutes. The concentrations of calcium and potassium ions (both given as the chlorides) were 0.02 N throughout. This concentration of calcium is known from the work of Cooil and Bonner (2) to inhibit essentially completely the auxin-induced growth of coleoptile sections. The same concentration of potassium, on the other hand, has no marked effect on the growth rate of coleoptile sections but does antidote the growth inhibiting effect of 0.02 N calcium solution (2). Indoleacetic acid (IAA) was used as the auxin throughout and given in a concentration of 2×10^{-5} M.

After the desired incubation interval the mechanical deformability of the coleoptile tissue is determined under conditions of static external load by use of the device shown in figure 1. In this machine, the coleoptile is secured at one end and loaded at the other with

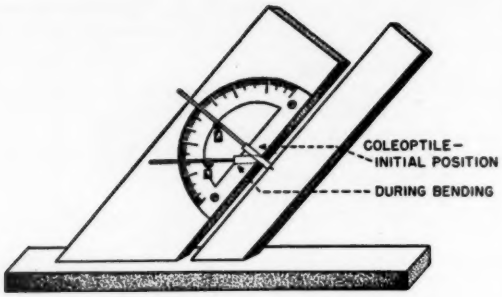

FIG. 1. Device for subjecting Avena coleoptile sections to an external load and for following the course of the resulting deformation.

a standard weight. Rate of bending of the coleoptile is then determined. The weight is next removed and rate and extent of recovery measured. The coleoptile section is first secured to the bending machine by impaling it on a fine stainless steel needle of about 1 mm diameter. This needle is fixed so that it extends exactly 5 mm into the hollow interior of the coleoptile cylinder. A fine glass rod, weighing 75 mg and 5 cm in length, bearing a central loop, is next inserted into the tip end of the coleoptile section. The apical part of this glass rod projects upon a protractor on which the initial position of the coleoptile is noted. A weight of 220 mg is then suspended on the loop. The coleoptile rapidly bends (fig 1) and the course of the movement with time may be measured on the protractor. In order to avoid drying of the experimental material, the entire apparatus is covered with a moistened bell jar to keep the surrounding air saturated during the observations.

Figure 2 gives data on the time course of the deformation response of Avena coleoptiles which had been previously equilibrated with pure water. When the load is applied, the coleoptile rapidly bends. This initial deformation is followed by a slower deformation which continues at a near steady rate for several minutes. When the load is removed, the coleoptile returns toward its initial position although the recovery is not complete. The recovery is thus the reversible portion of the deformation and is a measure of the elastic properties of the cell wall. The rapid initial deformation corresponds in magnitude to the elastic portion of the total deformation. The plastic properties of the tissue may evidently be measured by the slope of the continuing deformation curve of figure 2. We have chosen, however, as a routine procedure, to use total irreversible deformation over a loading time of 10 minutes as the measure of plasticity. Thus each measurement involves estimation of the total angle of deformation caused by the load over a period of 10 minutes, the plastic deformation or residual angle after removal of the load, and by difference, the elastic deformation.

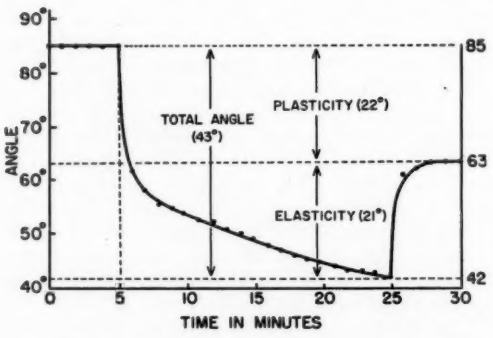

FIG. 2. Time course of Avena coleoptile deformation under load. Load (220 mg) imposed at 5 min. Ordinate: Angle of deformation measured on protractor.

Each coleoptile must be individually treated and its responses measured in the present device and this restricts the number of coleoptiles which may be used in a single experiment. Five replicate sections were therefore used in each part of each experiment, and each experiment repeated 2 to 5 times.

EXPERIMENTAL RESULTS

EFFECTS OF CALCIUM, MAGNESIUM AND POTASSIUM IONS ON THE MECHANICAL PROPERTIES OF THE TISSUE: The data of figure 3 A show that when coleoptiles are pretreated in a solution containing calcium ions, both elasticity and plasticity of the tissue are decreased. Coleoptiles were incubated for times ranging from 0 to 120 minutes in 0.02 N $CaCl_2$ solution. At the end of each incubation period, coleoptiles were removed to the bending machine and deformability determined as outlined above. Total deformability decreases rapidly during the first 30 to 60 minutes of incubation in calcium solution, approaching a constant low level with longer times. Both elasticity and plasticity are decreased (fig 3 A).

Pretreatment of coleoptiles with $MgCl_2$ solution (0.02 N) causes decreases of both elasticity and plasticity similar to those found to result from calcium pretreatment (fig 3 B). It appears therefore that divalent ions such as calcium and magnesium act to make the cell wall firmer and less susceptible to deformation under load.

When coleoptiles are pretreated in KCl solution (0.02 N), their total deformability is increased. This is due to an increase in elasticity since the plastic component decreases slightly as time of incubation in potassium solution is increased (fig 3 C). Potassium ions act, therefore, to make the coleoptile more deformable, particularly by making it more elastic.

When coleoptiles are soaked in an equinormal mixture of calcium and potassium ions, the effects of calcium ions on the mechanical properties of the cell wall are suppressed and the effects of the potassium ions predominate (fig 3 D). The effects of calcium and potassium ions on coleoptile plasticity as measured by the present direct method are thus quite similar to

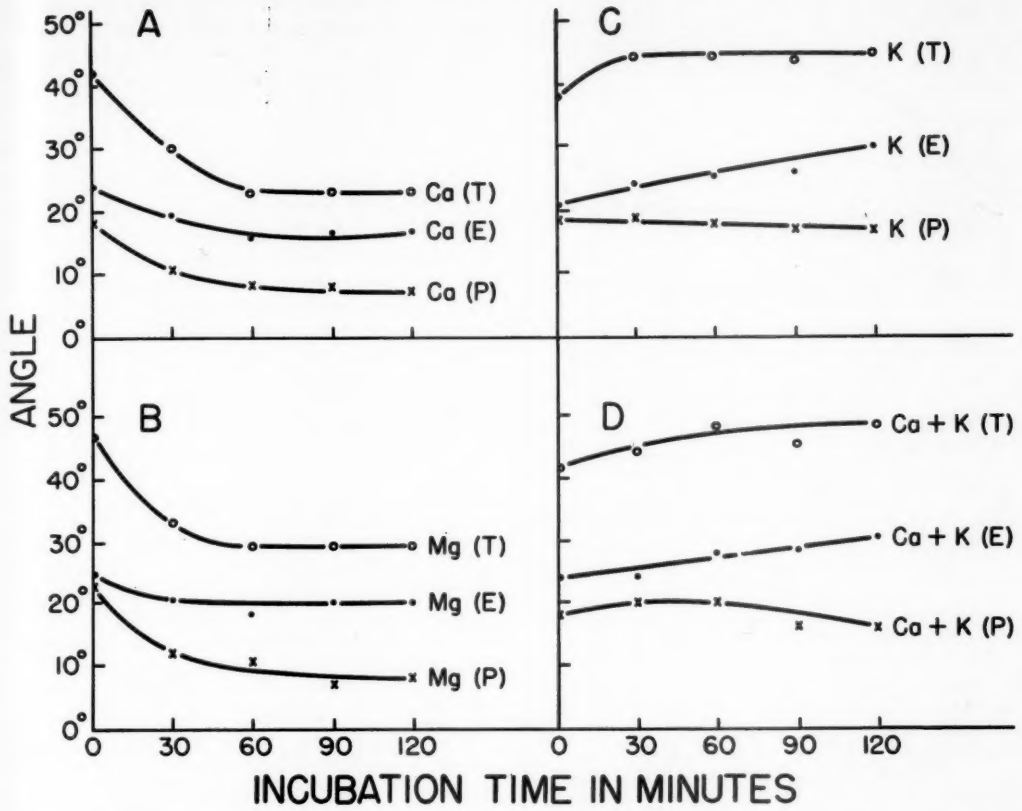

FIG. 3. Elasticity and plasticity of Avena coleoptile sections as a function of time of incubation in various salt solutions. A. $CaCl_2$, 0.02 *N*. B. $MgCl_2$, 0.02 *N*. C. KCl, 0.02 *N*. D. $CaCl_2$ and KCl, each 0.02 *N*. T refers to total angle of deformation, P to plastic angle and E to elastic angle.

the effects of the same ions on cell wall rigidity as measured by growth rate (Cooil and Bonner, 1956). In both cases calcium is inhibitory, potassium by itself has little effect but possesses the ability to antidote the effect of calcium.

The curves of figures 3 A to 3 D all indicate that the processes having to do with tissue deformability are quickly initiated when coleoptiles are placed in salt solution, and come to an equilibrium or steady state condition within about 60 minutes. The half time for attainment of this equilibration is approximately 15 to 20 minutes.

EFFECT OF IAA ON DEFORMABILITY: The data of figure 4 A concern the effect of IAA on coleoptile deformability. In confirmation of the results of Heyn (3), it is apparent that IAA increases the plastic component. This effect is a rapid one. A marked increase in plasticity can be detected within 30 minutes after transfer of the coleoptile to IAA solution. The time required to generate one-half of the maximum IAA-induced wall softening is of the order of 25 minutes. This is somewhat longer than the time required to achieve steady state elongation of coleoptile sections in IAA solution, which has been found to be not more than 10 to 20 minutes.

It has long been known that potassium salts act as cofactors in auxin-induced elongation of coleoptile sections (Thimann and Schneider (8)). The role of this ion is in part an osmotic one (5). In addition, however, potassium ions exert an influence on the mechanical properties of the tissue in the presence of IAA. This is shown by experiments in which coleoptiles were incubated in KCl, 0.02 *N*, together with IAA. Both plastic and elastic deformability are increased to a greater extent than is true for treatment with IAA or KCl alone (fig 4 B). When coleoptiles are incubated in IAA together wtih calcium chloride (fig 4 C) deformability remains essentially unchanged, even over a 120-minute period. Thus the stiffening effect of calcium ions appears to negate the plasticizing effect of IAA.

TRANSFER FROM POTASSIUM TO CALCIUM AND THE REVERSE: In order to find out more about the nature of the ion uptake responsible for the ionic effects on

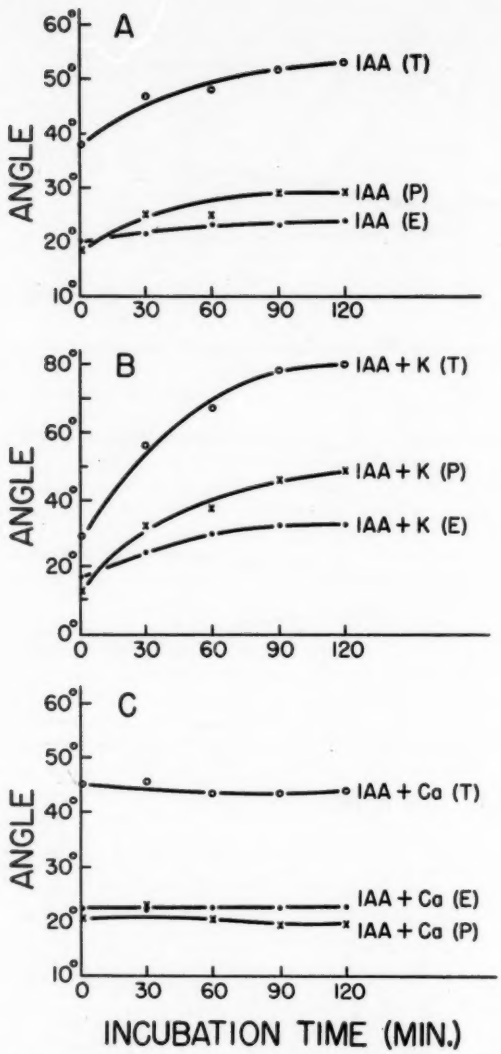

FIG. 4. Elasticity and plasticity of Avena coleoptile sections as a function of time of incubation in IAA solution and in the presence or absence of other salts. A. IAA alone. B. IAA in KCl, 0.02 *N*. C. IAA in $CaCl_2$, 0.02 *N*. T refers to total angle of deformation, P to plastic angle and E to elastic angle.

tissue deformability, coleoptile sections were subjected to equilibration in potassium containing solution and were then transferred either to water or to a solution containing calcium ions. The results (fig 5 A) show again that the elastic component of tissue deformability increases with time during incubation in the potassium solution. Coleoptiles transferred to water after equilibration with potassium retain the levels of plasticity and elasticity characteristic of tissue maintained in potassium solution and do not return to the initial levels characteristic of water equilibrated sections. When such potassium treated sections are transferred to calcium solution, however, the elasticity and plasticity of the tissue both decrease rapidly to the levels characteristic of calcium treated coleoptiles as is shown in figure 5 A.

Figure 5 B gives the results from experiments in which coleoptile sections were first incubated in calcium solution until equilibration had been attained with respect to elasticity and plasticity. These coleoptiles were then transferred to potassium solution or to water. Levels of elasticity and plasticity remain essentially constant after transfer of the tissue to water.

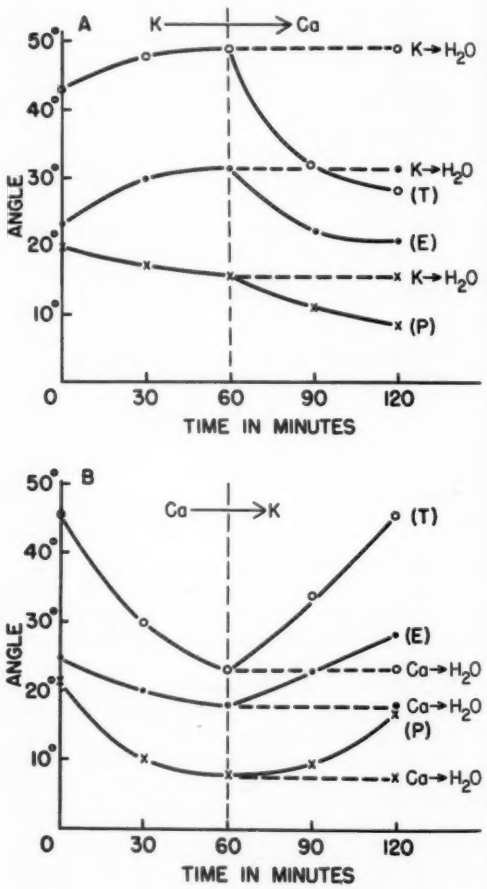

FIG. 5. Changes in the mechanical properties of Avena coleoptile caused by transfer of sections from KCl to $CaCl_2$ solutions and vice versa. A. Transfer from KCl solution to water ($K \rightarrow H_2O$) or to $CaCl_2$ solution. B. Transfer from $CaCl_2$ solution to water ($Ca \rightarrow H_2O$) or KCl solution. T refers to total angle of deformation, P to plasticity and E to elasticity.

When the coleoptiles are transferred from calcium solution to potassium solution, however, plasticity and elasticity increase rapidly to the levels characteristic of tissue incubated in potassium containing solution. The rate of equilibration of the tissue after transfer from one salt solution to a second is approximately equal to the rate of equilibration of tissue after transfer from water to salt solution.

The ions which influence the mechanical properties of the cell wall are therefore easily exchangeable with other external ions. They are not, however, readily lost to water. The effective ions behave in this respect as do exchangeably bound ions.

Discussion

This work has been done in order to find out something about the properties of the materials in the Avena coleoptile cell wall which control cell wall plasticity. It has been shown that wall plasticity is greatly influenced by the nature of the cations to which the tissue has been subjected. The effective ions appear to be exchangeably bound by the tissue. Thus calcium ions, which reduce cell wall plasticity, do so by interacting with the tissue in such a way that they are subsequently not lost to water but are lost to solutions of for example K ions. That Avena coleoptile tissue does possess the property of exchangeably binding calcium ions has also been shown directly by Cooil and Bonner (2) with the aid of labeled Ca^{45}, who further found that the time required for consummation of the exchange equilibrium is of the order of 30 minutes. The exchangeable binding of labeled calcium by coleoptile tissue possesses characteristics which are similar to those of the process by which calcium decreases cell wall plasticity. Thus not only is the latter process controlled by ions which are held in an exchangeable form, but in addition, the rates at which the two processes approach equilibrium are similar within the limits of precision of the measurements. These facts suggest then that the calcium ions which are bound in such a manner as to decrease cell wall plasticity may be the same ions whose exchangeable binding is detected and measured by the use of Ca^{45}. This hypothesis, if correct, would make possible an approach to the next question, namely, what constituents of the tissue bind the calcium ions which decrease cell wall plasticity? Evidently the material which binds calcium ions need not be directly dependent on the metabolic activity or structure of the cell since it has been found in the course of the present work that dead coleoptile tissue, killed by 5 hours incubation under argon (5), possesses 80 to 90 percent of the calcium exchange capacity of living tissue. That it may be the cell wall itself which contains the cationic binding groups under consideration is suggested by the fact that pectic materials which are major components of the Avena coleoptile cell wall possess the power of exchangeably binding cations. These facts suggest a picture of the coleoptile cell wall as made up of a network of pectic chains cross linked at intervals by ionic bonds in which calcium ions bind carboxylate ions of adjacent chains. This cross linked structure would correspond to the more rigid and less plastic state of coleoptiles equilibrated with calcium ions. Replacement of calcium ions by potassium ions would weaken such ionic interaction between pectic chains and yield the more plastic coleoptile characteristic of potassium equilibrated tissue. The cell wall of the coleoptile contains cellulose and other constituents in addition to pectic materials. These materials may well play their own roles in the growth process. That the pectic materials are of particular importance in the response of the wall properties to calcium and potassium ions is however suggested also by the parallel between the ionic effects on cell wall properties and effects of the same ions on pectic materials in vitro. Thus, solutions of pectic or pectinic acids become so extensively cross linked upon the addition of calcium that the solution sets to a gel. Potassium ions are without this effect since solutions of potassium pectate or pectinate remain in the sol form. It is of interest to note that appropriate experiments done during the course of the present work have shown that the calcium ions of Ca^{45} pectate gels are readily exchanged for potassium ions.

Summary

1. This investigation concerns changes in mechanical deformability of Avena coleoptile tissues under the influences of Ca and K ions and in the presence or absence of auxin (IAA). Deformability under applied load in a coleoptile bending machine has been used to measure the elastic and plastic properties of coleoptile cell walls.

2. When coleoptiles are pretreated in Ca or Mg solution, both elasticity and plasticity of the tissue is decreased strikingly. The divalent cations act to make the cell wall firmer. K ions, on the other hand, act in such a way as to make the coleoptile softer and more elastic.

3. IAA pretreatment increases the deformability of the coleoptile cell wall, primarily by increasing plasticity. The addition of K ions to the IAA solution augments the softening and plasticizing effects of IAA on the cell wall. Ca ions repress the plasticizing effect of IAA on the cell wall.

4. When Ca treated coleoptiles are transferred to K solution, or vice versa, the mechanical properties of the cell wall gradually become those appropriate to the new solution. The ions which exert the cell wall effects appear to be exchangeable with other external ions, and are not readily lost to water.

5. The kinetics of the exchangeable binding and release of labeled Ca by Avena coleoptiles are similar to the kinetics of the effect of calcium on the properties of the cell wall. It is possible that the ions bound exchangeably to the cell wall are those which affect its physical properties.

6. It is known that the physical properties of pectic materials are influenced by calcium and potassium

ions in much the same manner as are the physical properties of the cell wall itself. This suggests that pectic materials of the coleoptile cell wall may be of importance in determining the mechanical deformability of the structure.

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EFFECT OF GALACTOSE ON GROWTH AND METABOLISM OF AVENA COLEOPTILE SECTIONS[1,2]

LAWRENCE ORDIN and JAMES BONNER

Kerckhoff Laboratories of Biology, California Institute of Technology, Pasadena, California

During the course of investigation of the effect of various osmotically active solutes on Avena coleoptile section growth, it was found that galactose is more inhibitory to such growth than are other solutes in general. Mannitol for example is essentially without effect on section growth in concentrations up to 0.02 M, but galactose is highly inhibitory at this concentration. The inhibition caused by galactose is thus not due to the osmotic properties of the substance. Earlier Burström (2) found galactose to inhibit the growth of wheat roots. He indicated indirectly that galactose is respired but not apparently used synthetically, i.e., not assimilated into structural components. Thimann (11) also found that galactose inhibits growth of pea stem sections but he supposed galactose to be metabolically inert.

The galactose-induced growth inhibition of Avena sections has now been further investigated. The question is posed; is the inhibition exerted on energy producing enzymatic steps of respiration or is it exerted through an interference with cell wall metabolism, such as the production of cell wall components. The latter possibility is of inherent interest in connection with studies of auxin action since it has been shown that the primary act of auxin in cell elongation is upon the cell wall (3). Auxin increases rate of methylation of a cell wall fraction which possesses the properties classically ascribed to pectin (7).

The data presented here suggest that galactose inhibits cell elongation primarily by interfering with the synthesis of a cell wall fraction having the solubility properties of cellulose rather than by inhibiting respiration. The cellulose fraction of the cell wall is, however, as shown earlier (7), not influenced directly by auxin.

Methods

The material used in this investigation consisted of 5-mm sections cut 3 mm below the apex of Avena (var. Siegeshafer) coleoptiles. The Avena seedlings were grown as described earlier (6). All solutions were made up with redistilled water. Primary leaves were removed except in cases where elongation was measured.

Respiration measurements were made in the standard Warburg apparatus by following oxygen uptake. These measurements were made over a three-hour period.

Cell wall metabolism was studied by incubating sections in galactose-1-C^{14} or uniformly labeled glucose-C^{14} and by analyzing the cell walls as described by Ordin, Cleland and Bonner (7).

Aliquots of the hot water (pectin), hot acid (protopectin) and hot oxalate (pectate) extracts were plated on copper planchets. The alkali soluble fractions (hemicellulose and non-cellulosic polysaccharides) were neutralized with 0.5 N and 9 N H_2SO_4 before plating. The planchets were counted with a micromil window counting tube using Q gas. The final residues (cellulose) were counted with a thin-window GM tube. All counts are corrected to infinite thinness. The counts incorporated are expressed on a 10-mg cell wall basis.

The amount of radioactive substrate taken up by the tissue varied somewhat from experiment to experiment. Therefore a calibration experiment was done in which the extent of incorporation into each tissue component was determined as a function of

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amount of labeled substrate supplied and taken up. The calibration curves so obtained have been used to convert all of the data to incorporation per unit absorption of substrate. The data are expressed as counts per minute incorporated per 80,000 cpm of substrate absorbed.

The variability of Avena coleoptile section growth rates under the present conditions has been considered by Cleland and Bonner (3). In general growth increment differences greater than 0.06 mm are significant at the 5 % level. Variability in the measured incorporation of metabolite into tissue fraction is greater and differences in such incorporation of less than 10 % are not considered in this paper as meaningful. Each experiment here reported has been repeated many times.

The galactose-1-C^{14} was a gift from the National Bureau of Standards and the glucose-C^{14} was purchased from the California Foundation for Biochemical Research. Incubation of homogenized tissue with the radioactive substrates, followed by extraction and analysis indicated very little contamination of cell wall fractions by adsorption or occlusion of substrate.

Results

Figure 1 gives the results of an experiment which examines the effect of galactose on section elongation. Sections were placed in either 0.01 M galactose or 0.01 M mannitol. Potassium free indoleacetic acid (IAA) was added at the point in time indicated by the arrow. It may be seen that there is a marked effect of galactose on auxin-induced growth. If the galactose concentration is increased to 0.09 M in the presence of IAA, sections elongate only 0.09 mm in 20 hours whereas in a control solution of 0.09 M mannitol, elongation is 0.94 mm.

The effect of these two concentrations of galactose on respiration was investigated in order to determine if galactose blocks the normal respiration of glucose.

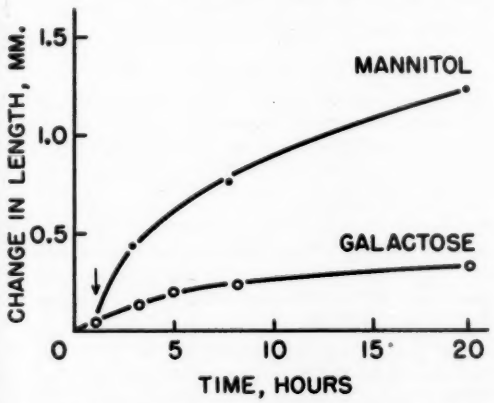

Fig. 1. Effect of 0.01 M galactose and of 0.01 M mannitol on the elongation of Avena coleoptile sections as a function of time in the presence of IAA. Five mg/l K-free IAA added at arrow, no buffer, final pH 6.1.

Table I

Effect of Galactose and of Mannitol Solutions on Oxygen Uptake of Avena Coleoptile Sections

Solutions	Conc	−IAA	+IAA
	M	*μl/20 sections × hr*	
Mannitol	0.09	13.0	21.0
Galactose	0.09	7.5	7.5
Mannitol	0.01	15.5	22.0
Galactose	0.01	24.0	27.0

Basal medium contains 5 mg/l K-free IAA, no buffer, initial pH 5.0 to 5.3.

Table I shows that 0.01 M galactose has no inhibiting effect on basal respiration but that 0.09 M galactose does inhibit respiration somewhat. The auxin induced increment in respiration is, however, almost eliminated by the lower concentration of galactose. This might be expected since the auxin induced respiratory increment accompanies elongation rather than the presence of auxin alone (cf Ordin, Applewhite and Bonner (6)).

The higher galactose concentration apparently interferes with respiration. That this might occur at the hexokinase stage is indicated by Hele (4) who has shown that phosphorylation of sugar mixtures by ATP in the presence of hexokinase yield lower rates than are obtained in the presence of single acceptors. Galactose in particular was found to be phosphorylated by intestinal mucosa at a slower rate than glucose. A mixture of the two sugars was phosphorylated more slowly than either alone.

The basis of this behavior is unknown but might account for the inhibition of respiration evident in table I at the higher galactose concentration. At the lower concentration however such inhibition does not appear to be a factor of importance.

A further test of the hypothesis that respiratory inhibition by galactose is not of primary importance

Table II

Effect of Various Treatments on Elongation of Galactose-Pretreated Avena Coleoptile Sections

Treatment	Elongation, mm *
0.08 M Glycol	0.26
0.08 M Mannitol	0.14
0.08 M Sucrose	0.54
0.08 M Glucose	0.52
0.045 M Tartrate	0.12
0.045 M H_3PO_4	0.27
0.045 M Malate	0.12
0.045 M Fumarate	0.26
0.045 M Citrate	0.17
0.045 M Succinate	0.28

Pretreatment: Incubation for 5 hrs in 0.01 M galactose, 0.0025 M potassium maleate, 3.5 mg/l IAA, pH 4.5.
Treatment: Incubation for 15 hrs in 0.01 M galactose and potassium salts or carbohydrate with maleate buffer, 3.5 mg/l IAA, pH 4.5.
* Elongation after 15 hrs.

TABLE III

$C^{14}O_2$ RESPIRED BY AVENA COLEOPTILE SECTIONS INCUBATED IN GALACTOSE-1-C^{14} OR GLUCOSE-C^{14}

SUBSTRATE	CPM/1000 CPM INITIAL SUBSTRATE
Glucose-C^{14}, 0.004 M	54.1
Galactose-1-C^{14}, 0.004 M	9.3

Initial specific activity: Glucose—182×10^6 cpm/mM, galactose—246×10^6 cpm/mM.

in the growth inhibition consisted in investigation of the effect of acids of the tricarboxylic acid cycle and of certain sugars on growth of galactose inhibited sections. Sections were pre-treated for 5 hours with 0.01 M galactose, 5 mg/l IAA and 0.0025 M potassium maleate pH 4.5. This was followed by 15-hour treatment in IAA, galactose and 0.045 M solutions of the acids or 0.08 M solutions of the sugars. The results are presented in Table II. Only glucose and sucrose had any effect in reducing the inhibition and even their effects were relatively small.

That galactose is indeed respired can be shown conclusively by incubating sections in a Warburg vessel with galactose-1-C^{14}. The CO_2 absorbed by the center well KOH was precipitated with $BaCl_2$ and counted as $BaC^{14}O_3$. Table III compares the amount of radioactive CO_2 respired with galactose-1-C^{14} or glucose-C^{14} as the substrates. The results are presented as cpm per 200 sections per 1000 cpm of initial culture solution. Each solution was 0.004 M with respect to the given sugar.

If it is assumed that both the galactose and the glucose are respired completely, then glucose is respired 6 times as rapidly as galactose. Under these conditions section growth is inhibited about 30 %.

Seegmiller, Axelrod and McCready (9) and Ordin, Cleland and Bonner (7) have shown that glucose gives rise to pectic substances in plant tissue. Because of the structural relationship between galactose and the pectic substances, experiments were performed to study the comparative incorporation of the two radioactive substrates into the various cell wall fractions.

Table IV gives the results of an experiment in which sections were incubated in 0.001 M radioactive galactose or glucose. For purposes of comparison it is assumed that the hexoses enter the cell wall fractions intact, i.e., without being dissimilated and reorganized. This concentration of galactose is non-inhibiting to growth. It may be seen that there is relatively more activity in the hot water soluble fraction (pectin) with galactose as substrate than with glucose, but that relatively less activity goes into the residue (cellulose) and hot acid soluble fractions (protopectin) with galactose as substrate. In fact, the differences between the two substrates are such that all fractions except cellulose and protopectin are more radioactive with galactose as substrate than with glucose as substrate. This is particularly marked for cellulose. It may be noted that auxin treatment causes an increase in the amount of galactose carbon incorporated into cellulose. Although in this particular experiment auxin does not cause a significant increase in cellulose activity with glucose as substrate, other experiments indicate that when cell wall expansion occurs, incorporation of glucose-C^{14} into cellulose is increased significantly by auxin treatment. This has also been shown by Boroughs and Bonner (1). The effect of IAA upon the pectin fraction is small both for the galactose and glucose cases. The small effects have been shown, in the glucose case, to be due entirely to the effects on a saponifiable substituent, presumably methoxyl (8).

Although the data imply interconversion of galactose to glucose, at least for the formation of cellulose, they also imply that this conversion is slow. That cellulose synthesis is essential to continued growth is indicated by the work of Wardrop (12) and of O'Kelley and Carr (5). The possibility may be considered therefore that galactose inhibits growth by being not

TABLE IV

INCORPORATION OF C^{14} FROM GALACTOSE-1-C^{14} OR FROM GLUCOSE-C^{14} INTO CELL WALL FRACTIONS OF AVENA COLEOPTILE SECTIONS IN THE PRESENCE AND ABSENCE OF IAA *

CELL WALL FRACTION	GALACTOSE		GLUCOSE	
	−IAA	+IAA	−IAA	+IAA
	Cpm/10 mg dry cell wall × 80,000 cpm absorbed by 150 sections			
Hot water soluble	833	938	575	624
Hot 0.05 *N* HCl soluble	7,725	8,390	13,300	11,050
Hot 0.5 % ammonium oxalate soluble	198	271	252	169
NaOH soluble, 4 %	2,210	2,345	2,370	1,560
NaOH soluble, 17.5 %	688	1,384	295	321
Residue	578	701	1,360	1,375
Totals	12,232	14,029	18,152	15,099

* Conc IAA used = 3.5 mg/l.

Initial specific activity of culture solutions: Galactose—142×10^6 cpm/mM, glucose—117×10^6 cpm/mM.

TABLE V

EFFECT OF 0.004 M GALACTOSE OR 0.004 M GLUCOSE ON INCORPORATION OF C^{14} FROM GLUCOSE-C^{14} INTO CELL WALL FRACTIONS OF AVENA COLEOPTILE SECTIONS

CELL WALL FRACTION	GALACTOSE	GLUCOSE
	Cpm/10 mg dry cell wall × 80,000 cpm absorbed by 150 sections	
Hot water soluble	453	528
Hot 0.05 *N* HCl soluble	2,270	3,040
Hot 0.5 % ammonium oxalate soluble	84	111
NaOH soluble, 4 %	1,204	968
NaOH soluble, 17.5 %	352	187
Residue	194	681
Totals	4,557	5,515

The culture solutions contained 3.5×10^5 cpm per 3.5 ml of C^{14}-labeled glucose in both cases; incubation for 3 hrs.

only itself a relatively poor substrate for cellulose synthesis but also by inhibiting glucose utilization for this purpose. An experiment was carried out to determine the effect of galactose on incorporation of glucose-C^{14} into the cell wall. To unlabeled glucose or galactose solutions, each 0.004 M, equal amounts of labeled glucose were added. Table V shows that the presence of galactose slightly inhibits incorporation of glucose carbon into pectic substance and slightly increases incorporation into the 4 % NaOH soluble fraction (hemicelluloses) and the 17.5 % NaOH soluble fractions (non-cellulosic polysaccharides). Most remarkable, however, is the fact that incorporation of glucose carbon into cellulose is severely inhibited by the galactose. This experiment was carried out in the absence of IAA to reduce artifacts due to elongation. Thus galactose not only is poorly utilized in cellulose synthesis itself but also inhibits glucose utilization in formation of this fraction of the cell wall. The inhibition is even greater than the growth inhibition under these circumstances.

Discussion

In contrast to Burström's finding that galactose is not used synthetically, it has been shown above that in the Avena coleoptile, galactose carbon is incorporated into the cell wall. Galactose is respired, as found by Burström, but not as rapidly as is glucose. Most noticeable is the fact that galactose carbon is not incorporated into cellulose as rapidly as is glucose. In low concentrations of galactose, auxin treatment causes radioactive galactose carbon to be incorporated more rapidly into cellulose than in the absence of auxin. Galactose also inhibits incorporation of radioactive glucose into cellulose. These lowered rates of incorporation are noticeable even when growth inhibition is small or nil.

Respiration is unaffected by growth inhibiting concentrations of galactose and the Krebs cycle acids are completely ineffective in overcoming the growth inhibition. Respiratory effects are not therefore involved in the inhibition of growth.

The depression of cellulose synthesis caused by galactose could be a factor of importance in limiting growth, albeit of a secondary nature as far as the auxin mechanism of elongation is concerned. Wardrop (12) has shown that as Avena coleoptiles grow the cellulose microfibrils tend to disperse. New microfibrils are, however, interpolated over the entire surface. This means that new synthesis does occur, a conclusion supported by the isotope incorporation data presented here as well as that of Boroughs and Bonner (1). O'Kelley and Carr (5) have found that the density of microfibrils appears unchanged in cotton fibers as they elongate suggesting that in this case synthesis keeps pace with elongation.

It would be of some interest to trace the route of galactose interference in cellulose synthesis more closely since this might help to illuminate the mechanism of cellulose biosynthesis.

Summary

Galactose at a concentration of 0.01 M inhibits elongation of Avena coleoptile sections quite markedly without affecting non-auxin induced respiration.

In lower concentrations which permit growth, carbon-14 derived from galactose-C^{14} is incorporated into all cell wall constituents except cellulose and protopectin as readily as in carbon-14 from glucose-C^{14}. Incorporation of carbon-14 from galactose-C^{14} into cellulose is markedly slower than is incorporation of carbon-14 from glucose C^{14}.

Galactose at 0.004 M has little effect on the incorporation of carbon-14 from glucose into cell wall fractions other than cellulose. Incorporation of glucose carbon into cellulose is strongly repressed by the presence of galactose.

Galactose is respired to carbon dioxide but at a lower rate than is glucose.

It is suggested that galactose specifically inhibits cell elongation by interfering with cellulose synthesis.

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METHYL ESTERIFICATION OF CELL WALL CONSTITUENTS UNDER THE INFLUENCE OF AUXIN[1,2]

LAWRENCE ORDIN, ROBERT CLELAND AND JAMES BONNER
KERCKHOFF LABORATORIES OF BIOLOGY, CALIFORNIA INSTITUTE OF TECHNOLOGY, PASADENA, CALIFORNIA

It has been shown in an earlier communication (11) that auxin increases the amount of incorporation of carbon-14 derived from methyl labeled methionine into a fraction of the cell wall of Avena coleoptile sections which possesses the properties classically associated with pectin. Incorporation into this material represents but a small part of the total, the main part being into the protopectin fraction. Such incorporation is not significantly affected by the presence of auxin. The auxin effect on incorporation into the pectin fraction persists in solutions which contain sufficient mannitol to prevent elongation during the course of the experiment. C^{14} from glucose was also found to be incorporated into pectin more rapidly under the influence of auxin. Bennet-Clark (1) and others have also suggested that auxin causes an increase in the methyl content of pectic substances of cell walls of elongating tissue. The hypothesis may be formulated that methylation of the carboxyl groups of adjacent pectin molecules under the influence of auxin may be involved in the splitting of anhydride or calcium bridges which contribute to the mechanical properties of the cell wall.

The present investigation was undertaken to determine to what extent general metabolic inhibitors interfere with the incorporation of methionine methyl into the pectin fraction and to answer definitively the question of whether methyl carbon incorporation accompanies only net pectin synthesis or whether such incorporation occurs at a more rapid rate than net synthesis and is therefore to be regarded as methyl ester turnover. It will also be shown below that esterification of pectin by methyl derived carbon is an auxin controlled reaction.

MATERIALS AND METHODS

The material for the experiments described below consisted of 5 mm sections cut 3 mm below the tip of Avena (var. Siegeshafer) coleoptiles grown in the conventional manner (10). Primary leaves were removed from all sections. Incorporation of the carbon of methionine or glucose into the cell wall of sections was allowed to occur by incubating sections in various solutions containing these substrates. One hundred fifty sections floating in 3.5 ml of solution were used for each treatment of each experiment. Solutions were buffered with 0.0025 M potassium maleate, pH 4.8; indoleacetic acid (IAA) was used at a concentration of 5 mg/l where indicated. Aliquots of the medium were counted before and after incubation to determine the amount of substrate taken up by the tissue.

After incubation for 3 hours at 25° C, sections were rinsed, dried on filter paper and ground with cold water in a mortar. The ground debris was handled and analyzed as described earlier (11). The varied cell wall fractions are separated on the basis of their solubilities and do not necessarily represent specific chemical entities. The names classically ascribed to each fraction will however be used in this paper for convenience.

All planchets were counted on an automatic sample changer using a micromil window tube and Q gas. All counts are corrected to infinite thinness.

The counts incorporated are expressed on a 10-mg dry cell wall basis. The amount of radioactive substrate taken up by the tissue varied somewhat from experiment to experiment. A calibration experiment was therefore done in which the extent of incorporation into each tissue component was determined as a function of amount of labeled substrate supplied and taken up. The calibration curves so obtained have been used to convert all of the data to incorporation per unit absorption of substrate by 150 sections (equivalent to 8 to 9 mg dry weight of cell wall). The data are expressed as cpm incorporated per 80,000 cpm absorbed for glucose; per 100,000 cpm absorbed for methionine.

Saponification of the methyl ester groups of the pectic fractions was accomplished by treatment with 0.1 *N* NaOH or NH_4OH at room temperature for one hour. No difference in the effectiveness of the two treatments was noted. Neither does it appear to be of importance whether the alkaline reaction mixture is neutralized before being taken to dryness for determination of radioactivity. As a check on the specificity of the alkaline saponification procedure, samples of the present pectin preparations were also demethylated by use of a highly purified pectin methyl esterase preparation. It was found that there is general agreement between the two methods so far as amount of radioactive material liberated by them in volatile form is concerned, although alkali always yielded somewhat more.

Incubation of homogenates with labeled substrate followed by analysis indicated little contamination of cell wall fractions by adsorption or occlusion of substrate.

RESULTS

The time course of incorporation of saponifiable methyl carbon into pectin and protopectin is given in

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figure 1. Unlike the remaining data this is expressed on a 10-mg dry wt basis without corrections for amount of substrate absorbed. The data show little effect of IAA upon amount of methionine absorbed. This situation is in marked contrast to that reported by Reinhold and Powell (14). These authors found that IAA increases glycine and glutamic acid absorption by sunflower hypocotyl sections by 40 % or more. The presence of IAA does however cause an increase in rate of incorporation of methionine methyl carbon. There is an initial lag in incorporation related perhaps to the time required to equilibrate the tissue with substrate. The effect of auxin in increasing rate of incorporation into pectin ester is however clearly apparent within 15 to 30 minutes of the initial lag. There is no appreciable effect of IAA upon incorporation into protopectin.

The radioactive pectin extracted from sections incubated with labeled methionine may be saponified for removal of the methyl ester groups. The residue from the saponification contains only 20 to 30 % of the original radioactivity, 70 to 80 % having been lost as a volatile material. The pectic acid liberated by the saponification may also be precipitated with alcohol as calcium pectate. The calcium pectate thus prepared was found to contain about 10 % of the radioactivity present in the original pectin fraction. This behavior is in contrast to that of pectin prepared from sections incubated with glucose-C^{14}. Such pectin loses only about 20 % of its radioactivity on saponification and about 80 % is recoverable in the purified calcium pectate. These observations confirm those reported earlier (11).

It was previously reported that auxin exerts an effect in increasing rate of methyl carbon incorporation into pectin even in sections prevented from elongating by incubation in 0.3 M mannitol solution. The data of figure 2 concern the rate of methyl carbon incorporation into pectin over the range of external osmotic concentration from very dilute buffer to just above incipient plasmolysis. It may be seen that increasing external osmotic pressure results in reduced incorporation into pectin esters over a 3-hour incubation. The absorption of methionine is not depressed by the increasing osmotic pressure. The effect of auxin on incorporation into pectin ester decreases slightly (percentagewise remains constant) as mannitol concentration increases, suggesting that a small part of the auxin induced incorporation may be an artifact of elongation. However, as Ordin, Applewhite and Bonner (10) have shown, elongation under these circumstances drops much more rapidly with increasing external concentration.

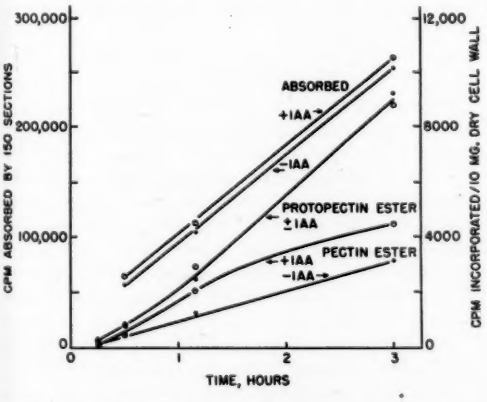

FIG. 1. Effect of IAA on absorption by Avena coleoptile sections and incorporation into esters of cell wall pectic substances of carbon-14 derived from methyl-C^{14}-methionine as a function of time. Potassium maleate pH 4.8, 0.0025 M, L-methionine, 0.00036 M, 5 mg L. IAA where indicated. The average specific activity of solution was 320×10^6 cpm/mM.

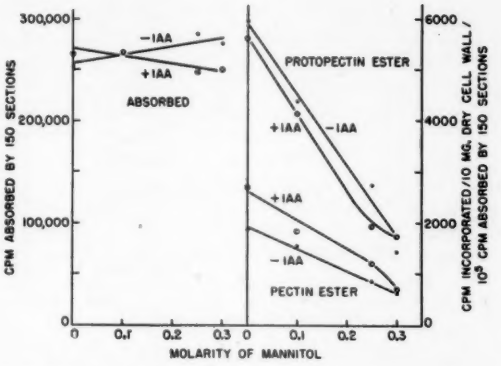

FIG. 2 A (*left*). Effect of external osmotic concentration on absorption by Avena coleoptile sections of carbon-14 derived from methyl-C^{14}-methionine in presence and absence of IAA. Conditions same as those of figure 1 except for addition of mannitol.

FIG. 2 B (*right*). Effect of external osmotic concentration on incorporation into esters of cell wall pectic substances of carbon-14 derived from methyl-C^{14}-methionine in presence and absence of IAA. Conditions same as in figure 2 A.

Although in the particular experiment of figure 2 the incorporation into protopectin ester is slightly less in the presence of auxin than in its absence, the data of figure 1 and of numerous other experiments indicate that there is no significant difference between the treatments.

It is clear that there is a substantial rate of methyl carbon incorporation into pectin in the absence of added IAA and that IAA merely increases this basal rate. The following experiment was done to find out to what extent the basal rate might be due to endogenous auxin. For this purpose sections were incubated with the antiauxin 2,4,6-trichlorophenoxyacetic acid (2,4,6-T) in the presence of C^{14}-labeled methionine. The data of figure 3 show that absorption of methionine was decreased by the higher 2,4,6-T concentrations. The incorporation of methyl carbon into protopectin ester was decreased even more. Incor-

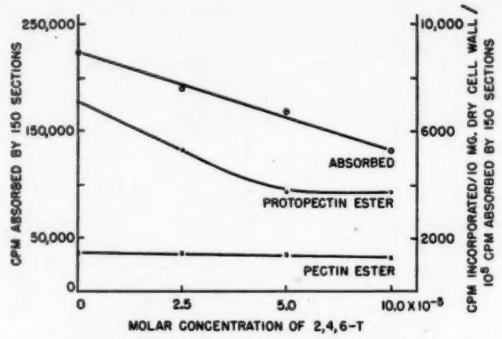

FIG. 3. Effect of various concentrations of 2,4,6-T on absorption by Avena coleoptile sections and incorporation into esters of cell wall pectic substances of carbon-14 derived from methyl-C^{14}-methionine. Conditions same as those of figure 1 except for absence of IAA.

poration into pectin ester however was essentially unaffected by 2,4,6-T. The antiauxin has then no effect on basal rate of incorporation into pectin. The basal rate is apparently not auxin controlled and in this is similar to the endogenous (no added IAA) rate of section elongation which is also not inhibited by 2,4,6-T.

It has been shown by Bonner (2, 3), Hackett and Thimann (6, 7) and others that 2,4-dinitrophenol (DNP) and anaerobic conditions inhibit auxin-induced cell elongation. These inhibitors also inhibit both the continuing and the auxin-induced increase in methyl carbon incorporation into pectin. The data of table I concern the effects of 2.5×10^{-5} M DNP or of argon on incorporation of methyl carbon into pectin and protopectin esters. The sections were pretreated with the inhibitors for one hour, after which time radioactive methionine was added and incubation continued for an additional 3 hours. The argon was supplied as described by Ordin, Applewhite, and Bonner (11). Table I shows that DNP reduces incorporation (corrected to equal methionine absorption) by over 90 %

TABLE I

INCORPORATION OF C^{14} FROM METHYL-C^{14}-METHIONINE INTO ESTERS OF PECTIC SUBSTANCES OF AVENA COLEOPTILE CELL WALL *

CELL WALL FRACTION	CONTROL		DNP **		ARGON †	
	−IAA	+IAA	−IAA	+IAA	−IAA	+IAA
	Cpm/10 mg dry cell wall × 10,0000 cpm absorbed by 150 sections					
Pectin	2020	3470	249	225	206	215
Protopectin	6250	8550	535	493	107	138

* Incubation, 3 hrs; methionine, 0.00036 M.
** Conc, 2.5×10^{-5} M.
† Bubbled 1 hr before and during incubation.
Average initial specific activity of culture solutions: 300×10^6 cpm/mM.

TABLE II

INCORPORATION OF C^{14} FROM UNIFORMLY LABELED GLUCOSE-C^{14} INTO PECTIC SUBSTANCES OF AVENA COLEOPTILE CELL WALL *

CELL WALL FRACTION	−IAA	+IAA
	Cpm/10 mg dry cell wall × 80,000 cpm absorbed by 150 sections	
Pectin	575	624
Protopectin	13,300	11,050
Pectin ester	114	181
Pectin non-ester	457	436

* Incubation, 3 hrs; glucose, 0.001 M.
Initial specific activity of culture solution: 117×10^6 cpm/mM.

and completely erases the auxin-induced increase. Argon treatment represses incorporation into pectin ester as much as does DNP but inhibits incorporation into protopectin ester to an even greater extent. In fact, in the argon treated sections, contrary to the usual situation, there is less incorporation into the protopectin ester than into the pectin ester. The absorption of methionine is inhibited by about 70 % by the DNP treatment and by about 60 % by argon treatment.

Earlier experiments (5, 11) have shown that the carbon of glucose-C^{14} is incorporated into cell wall constituents in the Avena coleoptile and that this incorporation is influenced by auxin. The data of table II show that glucose carbon is incorporated both into the saponifiable (ester) group of pectin and into the non-saponifiable residue, presumably into the polyuronide residues. That glucose can give rise to active one carbon units is known (15). The data of table II show in addition that the increased incorporation of glucose carbon into pectin in the presence of auxin, is due completely to the effect on incorporation into ester.

DISCUSSION

It is known that certain polyvalent cations, particularly calcium, inhibit both endogenous (no added IAA) and IAA-induced growth. Bennet-Clark (1) has found in addition that chelating agents which sequester such ions increase endogenous elongation of Avena coleoptile sections. The suggestion may be made therefore that methyl esterification may be the method by which polyvalent cation bridges between adjacent carboxyl groups of cell wall pectin are broken under the influence of auxin giving rise to increased cell wall plasticity. Calcium bridges between pectin chains are undoubtedly those of greatest importance but there is a possibility that pectin may be cross linked and that auxin may split anhydride bridges, these bridges being of the nature suggested by Kertesz (8). The exact compounds or enzymes to which IAA attaches itself to split such bonds are unknown. The bond splitting may either require methyl esterification as a primary part of the reaction or may require it to stabilize the split once made.

It seems clear that in the Avena coleoptile, bond splitting between pectin chains occurs as the result of methyl esterification with methionine as a methyl donor. Nance (9), on the other hand, has shown that IAA accelerates incorporation of acetate-1-C^{14} into the pectic substances of the pea stem and that part of this increase is due to incorporation of acetyl groups. Although acetyl groups may be involved in the cell wall metabolism of the coleoptile, their role has yet to be assessed.

It has been shown that a steady rate of methyl esterification is maintained in the coleoptile in the absence of auxin even when no elongation is taking place. Some synthesis of cell wall constituents is also maintained in the absence of added auxin. This can be detected by use of labeled glucose and such synthesis also occurs in the absence of elongation. The non-auxin induced esterification appears to accompany such synthesis. This basal esterification rate declines as external osmotic pressure is increased but the auxin induced increment in rate remains almost constant in absolute terms up to external osmotic concentrations near incipient plasmolysis. At osmotic concentrations higher than this the auxin effect disappears.

The small increase in rate of pectin synthesis under the influence of IAA which is indicated by the use of glucose-C^{14} as substrate is due to an effect of the auxin on a saponifiable fraction. It may be concluded that, in short term periods at least, auxin exerts little effect on net synthesis of pectin and that the effect of auxin on pectin esterification is the more important reaction. When sections elongate in optimal osmotic concentration the presence of auxin induces an increase in cellulose synthesis as has been shown by Boroughs and Bonner (5) with sucrose as substrate and by Ordin and Bonner (12) with galactose as substrate.

Perlis (13) also found that in elongating pea stems IAA enhances incorporation of acetate carbon into cellulose, protopectins and hemicellulose.

The antiauxin, 2,4,6-T, in the absence of IAA, and in the antiauxin concentration range, causes a slight depression of absorption of methionine and also causes a marked inhibition of incorporation of radioactivity into protopectin ester but does not inhibit incorporation into pectin ester. The cause of this inhibition is not known but it apparently has little to do with the endogenous elongation which may occur under these circumstances and which is not sensitive to antiauxin.

Anaerobic conditions and DNP both decrease incorporation as would be expected of an auxin sensitive reaction. As would be expected, these conditions inhibit absorption of the substrate, but when the data are calculated on the basis of amount absorbed, it becomes apparent that both auxin and non-auxin controlled incorporation of methionine methyl carbon are much more sensitive to metabolic conditions than is methionine absorption.

A question that remains unanswered is whether the increase in esterification mediated by auxin accompanies a conversion of protopectin to pectin which thereby loosens the cell wall, or whether the pectin fraction itself becomes less rigid, the protopectin remaining unaffected. That pectin ester may possibly be a precursor of protopectin ester in the synthetic process (with no direct relation to the auxin mechanism) is suggested by progress curves of incorporation. Pectin is as highly labeled as in protopectin in the early periods. Only over longer times does protopectin become the more labeled. Since protopectin constitutes most of the cold water insoluble pectic substance of the cell wall, it may behave as a sink for pectin. When glucose is used as the substrate a similar relation between pectin and protopectin is apparent over short time periods. Thus conversion of small pectic units to larger ones appears to be possible. Alternatively, it is possible that pectin is first labeled and that protopectin is labeled later merely because of differential reaction rates.

Efforts to demonstrate a direct conversion of pectin to propectin have yielded negative results. As indicated by Ordin, Cleland and Bonner (11) the presence of auxin slows loss of radioactive ester from pectin which has been previously ester labeled, but causes no significant change in protopectin. It is therefore virtually impossible to demonstrate a conversion of pectin to protopectin in vivo, if this does indeed occur. The retardation of loss of radioactive label from pectin caused by IAA is not in agreement with the findings of Bryan and Newcomb (4) who showed that IAA causes pectin methylesterase (PME) activity to increase in cultured tobacco pith before and during the cell enlargement phase. The action of PME should liberate carboxyl groups which would then be available for esterification by radioactive methionine. The present findings do not suggest that PME activity is increased by auxin in the Avena coleoptile.

Summary

IAA treatment of Avena coleoptile sections causes the methyl carbon of C^{14}-labeled methyl methionine to be incorporated into the pectin ester of the cell wall more rapidly than is the case in the absence of IAA. The effect is detectable within 30 to 60 minutes. IAA has no apparent effect on incorporation of methyl carbon into protopectin ester.

As the osmotic pressure external to the sections is increased, incorporation of methionine methyl into pectic esters decreases. The auxin induced increase in incorporation into pectin ester is however essentially unaffected up to external mannitol concentrations of 0.3 M.

The antiauxin 2,4,6-T, given in the absence of added auxin, has no effect on methyl carbon incorporation into pectin ester but depressed incorporation into protopectin ester. Incorporation of methyl carbon into pectin apparently possesses a basal rate which is not auxin controlled.

2,4-DNP and argon treatment both inhibit incorporation of methyl carbon into all cell wall fractions.

This inhibition is apparent even when corrections are made for effects on the amount of substrate absorbed.

The increased incorporation of glucose-C^{14} into pectin caused by auxin can be shown to be principally due to incorporation into saponifiable substituents, presumably methyl ester groups.

It is concluded that the data support the hypothesis that esterification of carboxyl groups of pectin is involved in the auxin mechanism of cell expansion.

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THE FORMATION OF STARCH AND THE BEHAVIOR OF ISOCITRIC ACID IN EXCISED LEAVES OF BRYOPHYLLUM CALYCINUM CULTURED IN DARKNESS[1]

HUBERT BRADFORD VICKERY

Department of Biochemistry, The Connecticut Agricultural Experiment Station, New Haven, Connecticut

In an experiment carried out in 1949 (1), confirmation was obtained of an earlier observation of the late Dr. G. W. Pucher that the starch content of leaves of *Bryophyllum calycinum* excised at daybreak may undergo a moderate increase during prolonged culture in water in darkness. It was noted at the same time that the isocitric acid content of the leaves decreased during the first 24 hours of the treatment by about 30 % of the amount initially present. This last observation has since been found to be unique. Isocitric acid is notably stable under most of the conditions, including long periods of culture in darkness, to which Bryophyllum leaves have been exposed in a variety of studies of the behavior of this substance. Only when the leaves are cultured in water for many hours under continuous illumination is there any indication that isocitric acid becomes involved in the metabolic reactions. In a recent experiment (2), for example, about 9 % of the isocitric acid disappeared after the leaves had been held for 74 hours under artificial lights. The rate of the reaction was notably sluggish and the total effect was small. Accordingly, it seemed essential to reexamine the behavior of starch and of isocitric acid under the conditions that were employed in the previous experiment.

In the early work of Dr. Pucher, the leaves had been supported in wire baskets in complete darkness in a sterilized metal cabinet where they were subjected to a continuous fine spray of water at 23° C. The surfaces of the leaves were thus continuously washed with water, the object being to minimize the possibility of contamination with microorganisms the growth of which might confuse the experimental results. Under these circumstances. the initiation of

[1] Received January 22, 1957.

rootlets at the indentations of the margins of the leaves was strikingly promoted, a point of some interest at that time. The possibility was overlooked, however, that the continuous fine spray of water might have washed most or perhaps all of the carbon dioxide out of the air of the cabinet and thus have interfered with the uptake of organic solids which is usually seen when Bryophyllum leaves are cultured in open troughs in a dark room the air of which is at about 50 % relative humidity. In the present experiment, therefore, two groups of identical samples were cultured in darkness, one in the metal cabinet under a spray of water at 24° C, the other in troughs in a dark room at 24° C and 50 % relative humidity.

Experimental Procedure

The ten samples of leaflets were collected by the statistical method (3) starting at 5: 00 A.M. (sunrise 5: 21 A.M.) June 2, 1954, the top 3 pairs of fully expanded pentafoliate leaves from 20 plants being used. The samples consisted of 60 leaflets, all plants and leaflet positions being equally represented in each. The initial control sample was placed in the drying oven at 6: 00 A.M. which was taken as the zero hour of the experiment, 4 samples were arranged in wire baskets with the petiole end down and placed in the cabinet under the water spray, and 5 samples were arranged with the petiole end immersed in water in the troughs in the dark room. All samples were in position by 6: 10 A.M. Single samples were removed from each set at the time intervals shown in table I, wiped dry and weighed, and then dried at 80° C for analysis. All had increased in fresh weight by from 3 to 6 % of their initial weight. The dried samples were equilibrated at 24° C and 50 % relative humidity in open wide-mouth bottles which were then closed and stored under the same conditions until the chemical analyses could be performed. The data for initial fresh weight, total and protein nitrogen and ash in table I show that the sampling error was acceptably small and that it could be assumed that the individual samples were essentially identical in chemical composition at the time of collection. Differences between the control sample which was dried at the start and the samples subjected to treatment can therefore be interpreted as quantitative effects of the treatment. The analytical methods employed have been described in recent publications (4, 5) with the exception of that for isocitric acid; this was determined by the highly specific enzymatic method of Grafflin and Ochoa (6). The enzyme preparation used is obtained by extracting an acetone powder of pig heart muscle with 10 parts by weight of 0.1 M phosphate buffer at pH 7.0. The extract is dialyzed against tap water for 16 hours (aconitase activity disappears) and is centrifuged and stored in the frozen state. It remains useful for many months. The analyses are carried out on the pooled titrated fractions from the Dowex 1 analytical column in which citric and isocitric acids are present. An aliquot is warmed with an excess of alkali to saponify any isocitric lactone which may have been formed during the preceding treatment, an essential precaution since the lactone does not react, and neutralized. At zero time suitable samples are added to cuvettes that contain an excess of triphosphopyridine nucleotide, manganous chloride and enzyme in buffer at pH 7.5. The maximal change in absorptivity at 340 $m\mu$ observed in the Beckman spectrophotometer after about 30 seconds and corrected by suitable blanks furnishes a measure of the amount of reduced triphosphopyridine nucleotide formed. This is equivalent to the amount of *d*-isocitric acid present.

Table I

Fundamental Data on Samples of Bryophyllum calycinum Leaves Collected by the Statistical Method

Sample no.	Culture period	Initial fresh wt per sample	Total N	Protein N	Ash
	hrs	*gm*	*gm/kgm initial fresh wt*		
1	0	239.87	2.18	1.77	12.52
2 *	6	239.94	2.19	1.78	12.62
3 *	12	242.73	2.20	1.79	12.38
4 *	18	246.50	2.21	1.74	12.48
5 *	30	241.77	2.21	1.77	12.54
6 **	6	237.51	2.26	1.84	12.87
7 **	12	241.50	2.14	1.82	12.61
8 **	18	239.85	2.24	1.78	12.77
9 **	30	236.20	2.22	1.78	12.88
10 **	35	240.59	2.23	1.75	12.78
Mean		240.65	2.21	1.78	12.65
S.D.†		± 2.83	± 0.03	± 0.03	± 0.17
C.V. (%)†		1.17	1.5	1.7	1.4

* Samples cultured in the spray cabinet.
** Samples cultured in troughs.
† S.D. = Standard deviation. C.V. = Coefficient of variation.

Experimental Results

The results of the determinations of starch are plotted in the upper part of figure 1. The leaves at the start contained 6.4 gm per kilo but, after culture for 6 hours in the spray cabinet, the starch had diminished to 4.7 gm per kilo. Subsequently it slowly increased so that after 30 hours 7.6 gm were present. Starch diminished in the leaves cultured in the troughs in exactly the same way, but the recovery was apparently slower and the final value after 35 hours was only 6.7 gm. It is important to consider what is meant by the term "starch" in this connection. The data record the behavior of a component of the leaves that is extracted from the finely ground tissue by perchloric acid and precipitated as a blue complex compound by iodine. The iodine complex is decomposed by an alcoholic solution of alkali, the insoluble white material obtained is isolated, hydrolyzed by 0.7 *N* hydrochloric acid and the glucose produced is titrated with a sugar reagent. This procedure constitutes an operational chemical definition of starch and includes

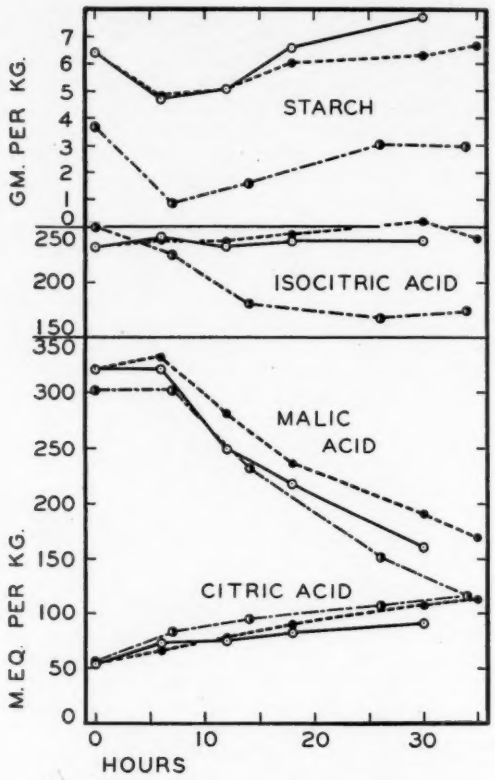

FIG. 1. Behavior of starch and of organic acids in excised leaves of *Bryophyllum calycinum* cultured in darkness under a continuous spray of water at 24° C (open circles, solid line) and in troughs in a room at 24° C and 50% relative humidity (filled circles, broken line). Data from a previous experiment in the spray cabinet (1) are plotted as half-filled circles connected by dash and dot line.

both amylopectin and amylose (7); no other well-known complex carbohydrate behaves in exactly this way.

The general behavior of the starch in the present experiment was almost precisely the same as that in the earlier one as is evident from the curve plotted for comparison (half-filled circles). The chief difference is that the starch content of the previous set of samples was initially lower being only 3.7 gm per kilo at the start; during the first 7 hours it diminished to 0.9 gm and then rose to 3.1 gm after 26 hours, subsequently remaining with minor fluctuations at a mean level of 3.2 gm for 175 hours. The earlier results as well as those of Dr. Pucher are thus amply confirmed.

Malic acid increased slightly for 6 hours in the leaves cultured in the troughs and then slowly decreased for the remainder of the culture period. After 6 hours in the spray cabinet, malic acid was at the same level as at the start but it then decreased in the same way as in the other group of samples. The behavior duplicated that observed in the earlier experiment remarkably closely (data plotted as half-filled circles), the rate of loss of malic acid being 6.7 meq per hour as compared with a rate of loss in that experiment of 6.9 meq per hour, these rates being calculated for the period of 24 hours in the one case and for 27 hours in the other from the point at which the loss began.

That there should have been a delay of 6 hours with the present set of samples before the reactions which bring about a loss of malic acid prevailed over those which lead to its formation could have been anticipated. Many previous observations have shown that malic acid increases in Bryophyllum leaves in darkness for a period of time that is moderately constant. When the leaves are first deprived of light, this acid increases for from 12 to 16 hours and then begins to diminish (2, 8). The present leaves were excised in dim light at sunrise after a night approximately 9 hours long and were then cultured in complete darkness. Malic acid remained constant or increased slightly for an additional 6 hours before diminishing. The period of time during which the synthetic reactions were in the ascendant was thus little if at all influenced by the act of excision of the leaves.

As is evident from the curves plotted in the mid-

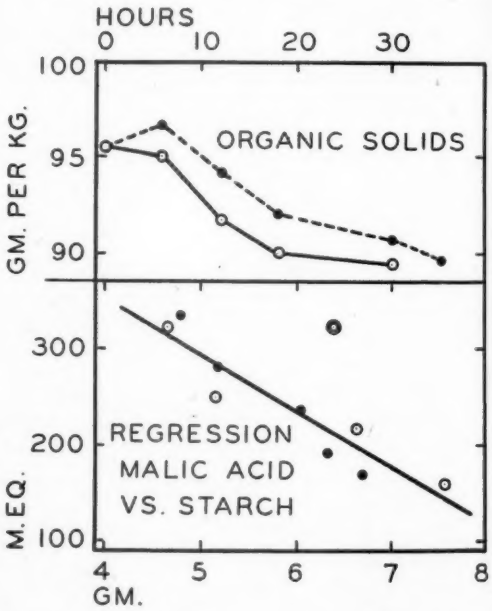

FIG. 2. *Upper part:* behavior of organic solids of *Bryophyllum calycinum* leaves cultured under the conditions given in the legend of figure 1 and plotted with the same symbols. *Lower part:* regression of malic acid upon starch in the same leaves for the observations between 6 and 35 hrs inclusive. Initial control sample shown as double circle, other symbols as in figure 1.

dle portion of figure 1, the behavior of isocitric acid in the present experiment was entirely different from that observed previously. Although the observation at 30 hours for the samples cultured in the troughs appears to suggest a slight increase, the data as a whole (10 observations) have a mean value of 239.8 ± 7.1 meq per kilo, the coefficient of variation being 2.9 %. Inasmuch as this variation includes both the sampling error and the analytical error, either of which may be as much as 2 %, it is clear that there is no evidence that a significant change in the quantity of isocitric acid occurred during the treatment of the leaves in the present experiment.

Citric acid increased continuously throughout the period of culture at nearly the same rate as it had in the previous experiment (data plotted as half-filled circles). The increase was slightly greater in the leaves cultured in the troughs than in those in the spray cabinet, but the general behavior was similar to that observed in many previous experiments.

The behavior of the organic solids of the leaves is shown in the upper part of figure 2. There was a small but significant increase in the leaves cultured in the troughs, an observation in conformity with the results of several previous experiments (2, 8, 10) carried out under similar conditions. Subsequently the organic solids slowly diminished owing to the effects of respiration. The leaves in the spray cabinet, however, did not increase in organic solids. The loss in the first 6 hours was small, but subsequently proceeded at about the same rate as in the other group of samples. It seems obvious that the conditions which obtained in the spray cabinet were such as to deprive the leaves of an exogenous source of carbon dioxide.

Discussion

The difference between the present observation of the constancy of the isocitric acid content of Bryophyllum leaves exposed to culture in darkness under a continuous spray of water and the result of the earlier experiment in which a prompt decrease occurred in leaves cultured under the same conditions raises an important question with respect to the position of this substance in the general metabolic scheme. The quantity of isocitric acid present changes little if at all during the normal diurnal variation in acidity of the leaves of this species (9, 10), at least to the extent that observations with excised leaves may be relied upon. Ordinarily this substance also appears to be unaffected during culture of the leaves in darkness, and it becomes involved only slowly and to a small extent under the stress occasioned by prolonged exposure of the leaves to light. There is no reason to doubt the accuracy of the determinations of isocitric acid in the earlier experiment since a method that has since been shown to be thoroughly reliable (11) was used. Accordingly, it is necessary to accept the result that a difference in behavior occurred.

The explanation of discrepancies such as this must necessarily be speculative, but there was one outstanding difference between the composition of the earlier samples and those used in the present experiment. The starch content of that set was the lowest of any that have been examined in this laboratory. Only one other set of samples has been seen which contained less than 5 gm per kilo of starch at sunrise, values between 5 and 10 gm and occasionally even higher being far more common. The starch content at sunrise is presumably a function of the effectiveness of the enzyme systems concerned with the acidification process that goes on during the night, and this in turn may depend upon such subtle factors as the nutritive condition of the plants and even the weather during the days preceding the collection of the samples. However this may be, it seems clear that the samples of the earlier experiment were unusual in their low content of starch. The prompt decrease of starch which occurred during the first few hours of the culture period shows that this substance was being drawn upon for some metabolic process, and apparently a level was soon reached at which other components of the tissue were required. Insufficient amounts of simple carbohydrates were present to supply the demand, for the glucose content increased from 1.6 to 2.2 gm per kilo during this critical period of 7 hours, and the sucrose and unfermentable sugar changed only a little. Under these circumstances, the requirements appear to have been met by isocitric acid, and this substance continued to be used for more than 14 hours or until the decomposition of malic acid was well under way. In the present experiments, by contrast, there appears to have been ample starch to supply the requirements of the metabolism, and isocitric acid was not drawn upon.

The observations therefore lead to the suggestion that isocitric acid may fulfill the role of a reserve or storage substance available for use in the general metabolism under certain situations one of which seems to have developed when the starch diminished to an unusually low level under the experimental conditions to which the leaves were exposed.

The increase in starch, which began at the time that the reactions which led to a net loss of malic acid assumed dominance, continued for only a relatively short time. The starch increased to approximately the level present at sunrise and then remained with little further change for many hours. This phenomenon has been encountered on several occasions (e.g. (2) and unpublished observations), and appears to be associated with the metabolism of malic acid. It would appear that there is a relationship between the quantities of starch and of malic acid such that a certain low level of starch is fairly promptly reestablished if for some reason it decreases below this point.

A statistical examination was therefore made of the data for starch and malic acid over the period when the starch content was increasing. The pooled data for the two groups of samples (9 observations) yielded a coefficient of correlation of -0.933 which is highly significant since $r = 0.898$ for $P = 0.001$ with 7 degrees of freedom.

A plot of the regression of the 9 determinations of malic acid against the corresponding determinations of starch during the period when the starch was increasing is given in the lower part of figure 2. The point at zero time is shown as a double circle and, as would be expected, bears little relationship to the other group of points. The regression line is expressed by the equation, Malic acid = − 58.3 (Starch) + 583. The slope shows the ratio of the loss of malic acid to the gain of starch and is expressed in the units meq/gm. It can be converted to molar units by the factor 162.1/2000 = 0.081 where 162.1 is the molecular weight of one unit of the starch molecule $(C_6H_{10}O_5)_n$ and 2000 is the relationship of meq of malic acid to moles. Accordingly, the equation implies that 4.7 moles of malic acid disappeared during the formation of 1 mole of starch. This result is in marked contrast to the observation that approximately 1 mole of malic acid disappears during the formation of 1 mole of starch in Bryophyllum leaves exposed to light (8), and suggests that the formation of starch in darkness under the conditions of the present experiment is, as it were, a side reaction in which some of the malic acid present becomes involved.

A more detailed statistical analysis of these data, for which we are indebted to Dr. C. I. Bliss of this Station, led to the conclusion that the separate regression lines for the data of the two groups of samples are not significantly different from each other at the 5 % level in either position or slope. Too few observations were available however to permit generalization upon either the identity of, or the difference between the effects of the two conditions of culture with respect to the behavior of starch and malic acid.

Summary

An earlier experiment in which excised leaves of *Bryophyllum calycinum* were cultured in darkness under a continuous spray of water has been repeated. The observation of a formation of starch from approximately the 6th to 30th hour of treatment has been confirmed, but the previous observation of a promptly initiated loss of a substantial proportion of the isocitric acid of the tissue has not; on the contrary, the level of isocitric acid remained constant. The difference in behavior is ascribed to the uniquely low level of starch present in the leaves of the earlier experiment. Under the stress of the experimental conditions, isocitric acid appears to have behaved as a reserve substance which was drawn upon to satisfy the demands of the metabolism during the early phase of the treatment. After the utilization of malic acid had become well established, loss of isocitric acid ceased. In the present experiment, in which the leaves contained an adequate supply of starch, this phenomenon did not occur.

Evidence is presented of a close correlation between the formation of starch and the loss of malic acid. However, the molar relationship between the quantities of reactants was such as to suggest that the formation of starch in darkness under the experimental conditions is of the nature of a side reaction in which a part of the malic acid became involved.

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ABSORPTION AND TRANSLOCATION OF 2,4-DICHLOROPHENOXYACETIC ACID AND P^{32} BY LEAVES[1,2]

GEORGE E. BARRIER[3] AND W. E. LOOMIS
DEPARTMENT OF BOTANY, IOWA STATE COLLEGE, AMES, IOWA

Foliar applications of nutrient minerals and the increasing use of systemic pesticides have served to reemphasize the importance of absorption and translocation in plant physiology. Studies of the movement of these materials have an immediate application value; at the same time they can aid in the solution of basic problems in plant behavior.

A number of workers (5, 12, 14, 24, 30) have stressed the importance of surfactants in increasing the effects of herbicides applied to leaves. Bryan et al (5) and Hauser (12) showed that the rate of absorption of 2,4-D as well as the total qualtity absorbed was increased by lowering the surface tension of sprays. Some of this effect is due to the better wetting leaves (12, 21) by solutions of low surface tension. However, leaves without surface wax deposits (23) show greater absorption of herbicides when surfactants are added, and additions far beyond those giving minimum surface tension give continued increases in toxicity (24). Weintraub et al (30), for example, found that surfactants increased 2,4-D absorption from alcoholic solutions, even though surface tension was not affected.

Bryan et al (5) and Hauser (12) showed that absorption of 2,4-D was accelerated by rising temperatures, indicating that chemical reactions are limiting the process at some point. The work of Bennett (2) suggests types of membrane changes that might be concerned in this response. Biddulph (3) and Swanson and Whitney (25) showed that P^{32} could be absorbed through leaf surfaces. The latter workers found, however, that this absorption was reduced by the single surfactant tried in their work.

The older work on translocation is reviewed by Curtis (8). More recent work, particularly as it applies to the mass-flow hypothesis, is reviewed by Crafts (6). Translocation of organic molecules, up or down, is normally limited to the phloem, while inorganic substances may move either in the xylem or phloem (17). Many workers find that translocation in the phloem is accelerated by temperature (4, 13, 25, 26), although complicating factors of respiration, fixation, etc., enter the picture at temperatures above about 25° C. Leonard (15) and others (18, 29) have shown that sugars may be exported from leaves against steep concentration gradients.

In the present research we have rechecked earlier experiments on the absorption of 2,4-D and have added experiments on the absorption of P^{32} by leaves and on the translation of 2,4-D and P^{32}. The radioactive element has been used as a check on the results with the herbicide.

MATERIALS AND METHODS

Soybean seedlings were the main experimental material. Uniform plants growing in pots were used when the unifoliolate leaves were mature but before expansion of the trifoliolate leaves had begun. Treatment with 2,4-D usually consisted of wetting 2/3 of one unifoliolate leaf on each plant with a solution containing 750 ppm of Na-2,4-D and 0.1 % of Triton B-1956. Penetration periods were controlled by washing the treated leaves thoroughly after a specified penetration time, typically two hours. The method of washing with a series of water and detergent solutions has been shown (12) to reduce residual 2,4-D on the leaf below that required for a significant effect on the plant. Translocation time was controlled by removing the treated leaf and its petiole after a specified time. Since significant penetration was obtained in two hours while significant translocation from the treated leaves required more than four hours (cf table II), it was possible to separate the effects of temperature, for example, on penetration and on translocation.

The effect of the 2,4-D absorbed (and subsequently translocated) before the treated leaves were washed, or absorbed and translocated before the treated leaves were removed, was measured as a reduction in the growth of the plant parts above the unifoliolate leaves. An experimental unit consisted of two, uniform, treated seedling plants in one four-inch pot. Ten replications, or 20 plants, were commonly used for each treatment. The minimum number of replications was six in a few experiments when a larger number of suitable plants was not available. All data were treated by analysis of variance. Values for least significant differences at the 5 and the 1 % levels ($LSD_{0.05}$ and $LSD_{0.01}$) are given in the tables whenever these values aid in the interpretation of the data. Plumule growth of soybean seedlings is variable under the best conditions and this variability is shown in the relatively large values required for least significant differences.

The P^{32} was obtained as a high specific activity solution (approximately 5 mc/ml) of the phosphate ion in a weak HCl solution at a pH of about 4.5. In general, 1 μc of P^{32} was dissolved in 10 μl of solution and applied with a micropipette to the central area of one unifoliolate leaf on each treated plants. Treated leaves were washed or removed at specified periods after applying the P^{32}, as for experiments with 2,4-D. P^{32} was measured in dried plant briquets by the counting method of Mackenzie and Dean (20). When

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[3] Present address: Grasselli Chemicals Department, Experimental Station, E. I. du Pont de Nemours and Co., Wilmington 98, Delaware.

TABLE I

EFFECTS OF 0.1 PERCENT WETTING AGENT ON THE ABSORPTION OF P^{32} WHEN APPLIED IN 20 μL OF SOLUTION

WETTING AGENT	ABSORPTION OF P^{32}	
	EXPT 1	EXPT 2
	cpm	*cpm*
None	7,968	6,421
B-1956	1,112	984
Tween 20	10,073	8,144
Dreft	2,592	2,028
Dodecyl alcohol	4,727	4,870
X-100	8,849	7,489

Expt. 1: $LSD_{0.05}$ = 3,961; $LSD_{0.01}$ = 5,403. Expt. 2: $LSD_{0.05}$ = 3,321; $LSD_{0.01}$ = 4,529.

the quantity of radioactive material of a specific tissue was small, or when the radioactivity was inconveniently high, the radioactive tissue was mixed in known proportions with non-active tissue before forming the briquets. Activity is reported for most experiments in counts per minute for comparable tissues receiving varying treatment. Variations in total activity with treatment, rather than specific activity, gave the information desired in these experiments.

RESULTS

Many workers have shown important effects of surfactants on the absorption of 2,4-D (cf 12 and 30). Swanson and Whitney (25) found no comparable effect on the absorption of P^{32} in an inorganic form. The results in table I show the variable effects of different wetting agents on P^{32} absorption. Some agents, as Triton B-1956, greatly reduced absorption, probably because of the formation of insoluble phosphorus compounds. None of the surfactants tested significantly increased the absorption of P^{32} by soybean leaves. If absorption had been through stomates (11) more effect from some of the wetting agents would have been expected.

TABLE II

ABSORPTION AND TRANSLOCATION OF 2,4-D BY SOYBEAN LEAVES

2,4-D, PPM	EXPOSURE, HRS	FATE OF LEAF AT END OF EXPOSURE	WT NEW GROWTH, GM
None	4	Washed	1.44
None	4	Removed	1.52
500	1	Washed	1.28
500	2	Washed	0.91
500	4	Washed	0.62
500	1	Removed	1.47
500	2	Removed	1.71
500	4	Removed	1.63
1000	1	Washed	1.22
1000	2	Washed	0.62
1000	4	Washed	0.94
1000	1	Removed	1.43
1000	2	Removed	1.60
1000	4	Remived	1.69

$LSD_{0.05}$ = 0.42; $LSD_{0.01}$ = 0.55.

TABLE III

EFFECT OF TEMPERATURE ON ABSORPTION OF 2,4-D DURING A 2-HR EXPOSURE

TREATMENT	TEMP, °C	WT NEW GROWTH, GM
None	15	3.86
None	30	4.35
2,4-D, 750 ppm	15	1.09
2,4-D, 750 ppm	30	0.60

$LSD_{0.05}$ = 0.72; $LSD_{0.01}$ = 1.09.

The general pattern of the absorption and translocation of 2,4-D is shown in table II. When the leaves were washed 1 hr after treatment to remove unabsorbed chemical, the total growth reduction 12 days later was not statistically significant. Reduction was apparent if the 2,4-D was left on the leaves for 2 hrs before washing. These figures indicate rates of penetration into leaves which subsequently remained on the plants so that translocation was limited only by absorption. When the treated leaves were removed at the end of the treatment period, none of the growth figures was lowered significantly, even with 4 hrs and 1000 ppm 2,4-D. Translocation of 2,4-D from these leaves was a slow process, and was not significant 2 hrs after toxic levels of the herbicide had penetrated into the leaves. In subsequent experiments with 2,4-D, 2 hrs was allowed for the absorption of 2,4-D and 24 hrs for translocation from the leaves.

A composite picture of the movement of P^{32} in a soybean plant somewhat larger than those generally used is given by figure 1. One μc of P^{32} was applied to one unifoliolate leaf after the first trifoliolate was partly unfolded. The treated leaf was washed after 2 hrs and the plant was allowed to grow for 1 week, then pressed, dried and radioautographed. The treated leaf was removed before making the exposure to avoid streaking the plate. P^{32} was abundant in the stem, roots and new leaves. It was nearly absent from the untreated unifoliolate leaf and also from the trifoliolate which was partly unfolded at time of treatment. Accumulation was most marked in the bud and in the root tips and branch root primordia. The reduced movement into older leaves is an indication

TABLE IV

EFFECT OF TEMPERATURE ON ABSORPTION OF P^{32} DURING A 2-HR EXPOSURE

TEMP	ABSORPTION OF P^{32}	
	EXPT 1	EXPT 2
°C	*cpm*	*cpm*
15	6,023	2,905
30	5,856	3,275

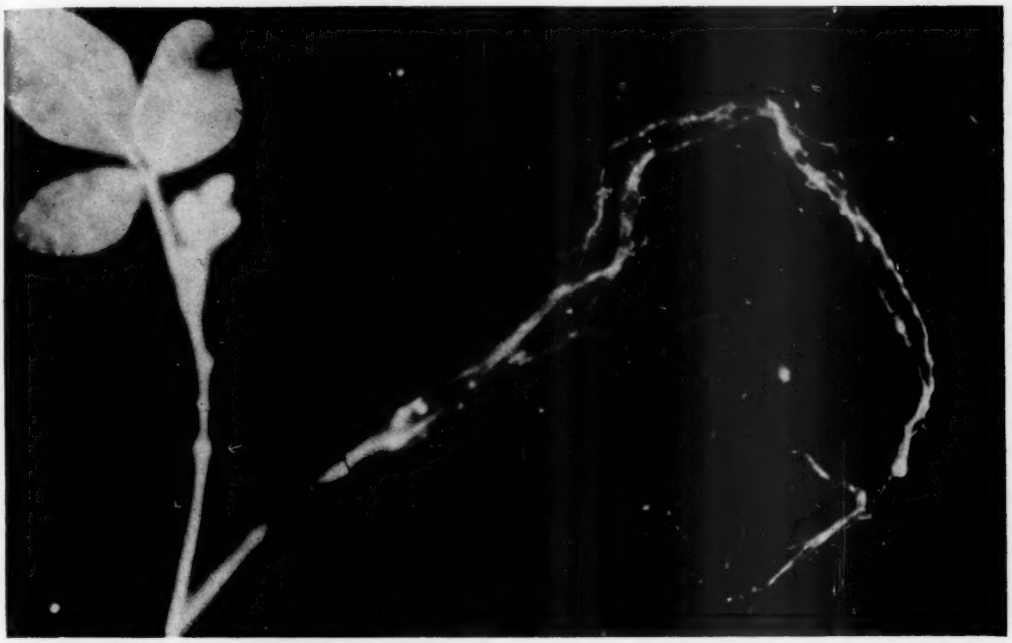

FIG. 1. One μc P^{32} was applied to one unifoliate leaf of a soybean seedling. The treated leaf was washed 2 hrs later, the plant allowed to grow for 1 wk, then pressed dried and radioautographed.

The treated leaf, containing 66 % of the absorbed P^{32}, was removed before autographing. Note near absence of translocation into leaves that were mature (unifoliolate) or partly expanded (first trifoliolate) at time of treatment.

TABLE V

EFFECT OF SUCROSE ON ABSORPTION OF 2,4-D BY CARBOHYDRATE DEPLETED PLANTS

TREATMENT	WT NEW GROWTH, GM
None	1.25
Sucrose, 5 %	1.31
2,4-D, 750 ppm	0.77
2,4-D + sucrose	0.88

that the phosphorus was largely changed to and remained in an organic form, hence did not rise from the roots in the transpiration stream. Exclusion from older leaves agrees with the findings of Leonard (15) and Loomis (18) that translocation of organic materials is polarly outward from older leaves.

The positive effect of temperature on absorption of 2,4-D is shown in table III. The *reduction* in growth at 15° C was 2.77 gm compared to 3.75 gm at 30° C. A temperature differential was maintained only during the 2-hr period immediately after treatment. The treated leaves were then washed thoroughly to remove unabsorbed chemical and the plants were grown together in the greenhouse for 12 days. This result agrees with those obtianed by Bryan et al (5) and Hauser (12), although the response was less striking in this experiment.

The absorption of P^{32} showed no temperature effect (table IV). The absorption of inorganic P^{32} thus differs from the absorption of an organic molecule in being unaffected by both wetting agents and temperature.

Added sucrose had no effect on the absorption of 2,4-D, table V. Light before, but not after, treatment increased absorption of P^{32}. It is not clear whether opening of stomates or electrcial charge of leaves (27) was involved in this effect on P^{32} absorption.

In contrast to the experiments on absorption, the addition of sucrose to carbohydrate depleted leaves increased the translocation of both 2,4-D and P^{32}

TABLE VI

EFFECT OF SUCROSE ON TRANSLOCATION OF 2,4-D FROM CARBOHYDRATE DEPLETED LEAVES

FATE OF TREATED LEAF AT END 24-HR DARK PERIOD	TREATMENT		WT NEW GROWTH
	2,4-D	SUCROSE	
	ppm	%	*gm*
Leaf attached	0	5	1.11
Removed	0	5	0.99
Removed	750	5	0.79
Removed	750	0	1.16

$LSD_{0.05} = 0.24$; $LSD_{0.01} = 0.33$.

TABLE VII

EFFECTS OF SUCROSE ON THE TRANSLOCATION OF P^{32} FROM CARBOHYDRATE DEPLETED LEAVES OF SOYBEANS

TREATMENT DURING EXPOSURE	SUCROSE	P^{32}
	%	*cpm*
Dark	0	749
Dark	5	4,791
Light	0	15,701

$LSD_{0.05} = 3{,}340$; $LSD_{0.01} = 4{,}683$.
All plants received 1 μc P^{32} in 10 μl of solution. All treated leaves removed after a 24-hr exposure.

(tables VI and VII). For 2,4-D the comparison between plants receiving 2,4-D, with and without added sucrose, was particularly striking. In the experiment with P^{32}, the effect of sucrose was just short of the 1 % level of significance. Light before and after application of the phosphorus gave an even larger effect, presumably because it resulted in a better carbohydrate supply within the leaf. Our results add P^{32} to the list of 2,4-D (9, 16, 22) and Co^{60} (11) the translocation of which appears to depend upon the sugar supply in the leaf. This effect of sugars on translocation has been claimed by Day (9) to indicate mass flow in the translocation of 2,4-D, with the herbicide swept along in a translocation stream of sugars. Work by Currier et al (7) raises a serious question of the mechancial feasibility of mass flow. It seems at least equally probable that a high level of carbohydrates may be required to supply energy or materials for some step in the loading (19) or movement of the chemicals. It is significant in this connection that a period of hours was required for the export of measurable quantities of 2,4-D or P^{32} from treated leaves. In contrast, Vernon and Aronoff (26) obtained export of radioactive sugars from soybean leaves within 5 minutes after supplying $C^{14}O_2$ in light.

A separation of the organic and inorganic fractions of P^{32} was made by the trichloroacetic acid method. Analyses showed that inorganic P^{32} applied to leaves was largely transformed to organic compounds within the leaf. Fang and Butts (10) have shown that 2,4-D may form organic combinations within the plant. If translocation is limited or essentially limited to some such combination, sugars could have a controlling effect without the introduction of the mass-flow hypothesis. The work of Wanner (28) suggests, however, that the translocated form of P^{32} is not a sugar phosphate.

TABLE VIII

EFFECT OF TEMPERATURE ON TRANSLOCATION OF 2,4-D DURING A 24-HR PERIOD

TREATMENT	TEMP, °C	WT NEW GROWTH, GM
Check	15	1.89
Check	30	1.86
2,4-D, 750 ppm	15	1.62
2,4-D, 750 ppm	30	1.22

$LSD_{0.05} = 0.23$; $LSD_{0.01} = 0.31$. $Q_{10} = 1.79$.

TABLE IX

EFFECTS OF TEMPERATURE ON TRANSLOCATION OF P^{32} DURING A 24-HR PERIOD

TREATMENT	TEMP	P^{32}	
		EXPT 1	EXPT 2
	°C	*cpm*	*cpm*
Treated leaves left on	..	2,878	2,133
Treated leaves removed at end of 24 hrs	15	450	353
" "	30	1,614	1,037

Expt. 1: $LSD_{0.05} = 1{,}004$. Expt. 2: $LSD_{0.05} = 507$. $Q_{10} = 2.21$ (av).

The effects of temperature on the rate of translocation from leaves are shown in tables VIII and IX. The Q_{10} for translocation of 2,4-D, calculated on the reduction of growth of plants treated at 15 and 30° C, was 1.79. This coefficient is in the range of processes limited by chemical reactions, and considerably larger than the value of 1.31 for the change in the viscosity of a 20 % sucrose solution over the same temperature

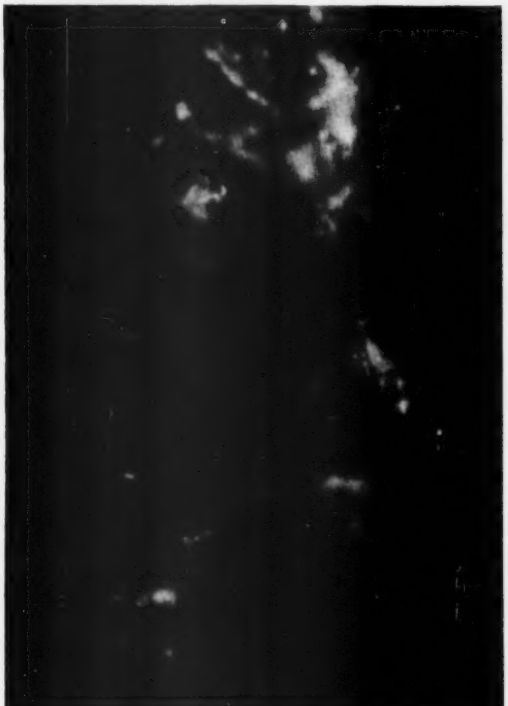

FIG. 2. Radioautograph of excised beet leaf blade washed and frozen 1.5 hrs after painting with P^{32} solution. Irregular penetration and little accumulation of P^{32} in veins.

FIG. 3. Radioautograph of beet leaf blade washed and frozen 6 hrs after painting with P^{32} solution. Heavier penetration than in figure 2 and marked loading of the radioactive phosphorus into the main veins.

range. In the experiments with P^{32} the temperature coefficients, Q_{10}, averaged 2.21, a typical coefficient for an enzymatically controlled chemical reaction. These responses and similar results obtained by others seem to establish the presence of some chemical reaction as the slow step in translocation from leaves. They do not distinguish between reactions changing the form of the translocated materials and respiratory reactions supplying energy for some step in the process.

For a comparison of absorption by leaves and roots, soybeans were grown in culture solutions until the unifoliolate leaves were mature. The culture jars were placed in water baths at 13 and 23° C and the solutions allowed to come to the bath temperatures. 2,4-D at 45 ppm was then added to the solutions and left for 24 hrs. The temperature coefficient for absorption of 2,4-D by roots was just over 1, as measured by subsequent growth of the plants, indicating basic differences in the absorption of 2,4-D by roots and leaves.

The work of Leonard (15) showing the extreme polarization of movement of sugars from sugar beet leaves suggested an experiment with P^{32} on this material. Solutions of P^{32} were painted uniformly over the detached blades of sugar beet leaves (approximately 100 μc per leaf). The blades were then held in moist chambers with the base of the cut midrib dipping in water. After varying periods the leaves were removed, scrubbed to remove surface P^{32} as thoroughly as possible, and radioautographs made of the fresh, frozen leaves at a temperatures of $-20°$ F. Experiments with dried leaves showed a pronounced tendency for equalization of P^{32} throughout the leaves as they dried.

The results of these tests are shown in figures 2 to 5. Figure 2 shows the radioautograph of a blade washed 1.5 hrs after applying the P^{32}, frozen, and radioautographed. Penetration was spotted, and the veins were generally low in radioactivity. The leaf in figure 3 was washed and radioautographed after 6 hrs and shows a very different condition. Penetration had been considerably greater, but, of greatest importance, practically all of the P^{32} in the effectively washed areas of the blade was collected in the main veins. Since these were detached blades, no translocation was involved, and the phenomenon is clearly that which we have called loading (19).

The accumulation of scattered P^{32} molecules into the veins of the leaf requires work, and we assume that respiratory energy is used. Figure 4 shows the accumulation of phosphorus in the veins of a blade normally supplied with carbohydrates and kept in diffuse light for 24 hrs after treating and before washing. Accumulation in all main veins was marked. A

FIG. 4. Loading in a beet leaf blade after 24 hrs. Most of the P^{32} was accumulated in the veins, even though no translocation occurred in this excised blade.

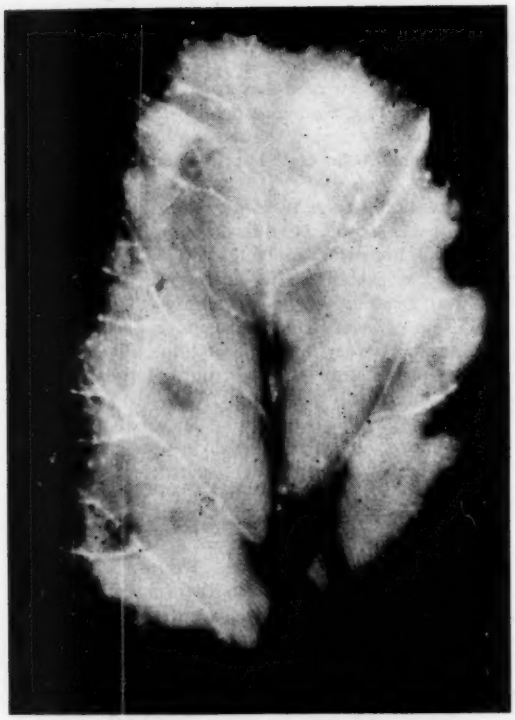

FIG. 5. General absence of loading in a blade low in available carbohydrates. Otherwise the same as figure 4.

paired leaf blade which was covered to exclude light before and after applying P^{32}, and thus to bring about carbohydrate depletion in the leaf, is shown in figure 5. Loading was greatly reduced, particularly in the midrib.

Loading, or some comparable reaction, has been indicated by experiments on polarization of transport from leaves. Leonard (15), Loomis (18) and Wanner (29) have shown that sugars may move out of leaves against diffusion gradients. Wanner found the sugar concentration of the phloem to be 20× that of the blade and to consist entirely of sucrose, whereas blade sugars were mixed sucrose and reducing sugars. Loading seems clearly to be a concept to be added to our knowledge of translocation, and is an additional complication in an already complicated situation.

SUMMARY AND CONCLUSIONS

The absorption of 2,4-D by leaves is increased by surface active chemicals and by increased temperature. The absorption of inorganic phosphorus showed neither of these responses. The results suggest differences in the mechanism of absorption of an organic molecule and an inorganic ion. Absorption of 2,4-D by roots of soybean plants was not accelerated by a temperature rise of 13 to 23° C.

Radioactive phosphorus pipetted onto one trifoliolate leaf was translocated throughout the plant. Sixteen percent of the P^{32} applied to a leaf had been absorbed in 2 hrs and could not be removed by washing. Of the absorbed P^{32}, 66 % remained in the treated leaf. The remainder was concentrated in the bud and young growing leaves, in the stem above the point of application, and in the roots. Leaves which were mature or considerably expanded at the time of treatment received relatively small quantities of the phosphorus.

Absorption of 2,4-D and P^{32} was not reduced by depletion of leaf carbohydrates. Translocation from the leaves, in contrast, was slowed or stopped. Translocation of both substances showed the temperature coefficients of a chemical reaction, with Q_{10} values on the order of 2. Absorption and movement of either 2,4-D or P^{32} from leaves in significant quantities required more than 2 hrs. Absorption alone required less than 2 hrs. Radioactive photosynthate may move out of soybean leaves within minutes after supplying $C^{14}O_2$ (26). This difference suggests that chemical or physical transformations are required before 2,4-D and P^{32} can be translocated through the phloem of soybean plants. Analyses of leaves treated with P^{32} showed that 80 % of the radioactive phosphorus was present in organic compounds after 24 hrs.

Six hours after painting detached beet leaf blades with P^{32} solution, a large percentage of the phosphorus was concentrated in the veins. Considerable areas of mesophyll were nearly free of radioactive material. This loading of the veins occurred in the absence of translocation from the blade but required a supply of available carbohydrate. Various observations on polar transport from leaves suggest that this loading reaction is a normal and important part of the translocation mechanism.

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GROWTH IN VITRO OF MARIGOLD AND TOBACCO TISSUE WITH NUCLEIC ACIDS AND RELATED COMPOUNDS[1,2]

ALBERT C. HILDEBRANDT, A. J. RIKER AND ELIZABETH MUIR
Department of Plant Pathology, University of Wisconsin, Madison, Wisconsin

With increased recognition of the importance of nucleic acids for growth, the roles of certain acids, of their individual component parts, and of some analogs have needed clarification both for normal and diseased growth.

Concerning normal growth, this interesting viewpoint may be mentioned. As a dicot stem matured, many cells as they differentiated might be in a "straight jacket" so that they could no longer divide unless released. In any case, the encouragement or inhibition of growth by common metabolic chemicals might be approached with tissue cultures.

Concerning diseased situations, tolerances shown by tissues might be critical. This is so whether abnormal amounts of metabolites have occurred through abnormal physiology or because of parasitism. Cellular responses have varied from the normal between such extremes as abnormal cell growth, injury, and necrosis. The encouragement or inhibition of growth by various common sources of nitrogen, carbohydrates, alcohols and organic acids, as well as tolerances for them, have been reported earlier (16).

Some relationships of the nucleic acids in the crown gall disease of plants were reviewed by Braun (1) and by Klein and Link (10). Certain correlations of these materials to cell enlargement, cell division and differentiation in isolated tissues of normal origin were analyzed recently by Skoog (18). Plant tissue that grew in vitro under controlled chemical and physical conditions provided excellent experimental material to examine the metabolic roles of some of these compounds.

Certain of the nucleic acid components added to

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the basal tissue culture media by various investigators have induced differences in morphology and rates of growth (8, 12, 13, 14, 19, 20).

However, less is known about the influences of these compounds on diseased plant growth. Virus tumor tissue of *Rumex acetosa* L. grown on media with supplements of nucleic acids and related compounds (15) were less responsive than were the tissues of normal origins. Growth of this tissue was enhanced by alkaline hydrolyzed ribonucleic acid (RNA) and decreased by alkaline hydrolyzed deoxyribonucleic acid (DNA). Marked inhibition was observed in media with supplements of adenine, adenosine or adenylic acid. Some evidence of stimulation occurred with guanine, uracil, xanthine or hypoxanthine. No evidence was recorded of induced morphological changes in the virus tumor tissues, such as those cited above for tissues of normal origin.

The purposes of the present paper were to determine the effects of certain nucleic acids and of their components on some plant tissue in culture. Attention was given to the influences of different concentrations on tolerance, stimulation, or inhibition and to relations with disease situations.

Materials and Methods

The marigold crown gall and the hybrid tobacco tissues were from stock cultures described earlier (4). Seed pieces with wet weights of about 20 mg were taken aseptically from cultures 35 to 42 days of age. Four seed pieces were spaced evenly on 50 ml of agar medium in a 6-oz prescription bottle set on its narrow side.

The nucleic acids and related compounds were used as received without additional purifications. D-Ribose was supplied by General Biochemicals, Inc., and the other compounds by Nutritional Biochemical Corporation. All compounds were soluble in hot water except DNA, RNA, guanine, xanthine and hypoxanthine. These dissolved when a few drops of 0.05 *N* NaOH were added to the solution. Concentrated stock solutions and a series of diluted solutions were prepared of the purines, pyrimidines and thiouracil. Appropriate amounts of these stock solutions were added to the basal medium for tobacco tissue (5) to give the desired concentrations. The medium was then adjusted to pH 6.0 and sterilized in the autoclave (15 pounds for 20 minutes). The concentrated D-ribose stock solution was sterilized by filtration and was added aseptically to the autoclaved basal medium.

Thymine, uracil and D-ribose each, respectively, were added to the medium to give concentrations of 0.04, 0.4, 4.0, 40 and 400 mg per liter. Xanthine, hypoxanthine, guanine, cytosine, adenine and thiouracil each, respectively, were added to the basal media to give concentrations of 0.01, 0.1, 1.0, 10 and 100 mg per liter. The concentration ranges for DNA and RNA were extended to 4 gm per liter. With tobacco tissue RNA was also used at 8 and 16 gm per liter. D-Ribose was tested both with and without sucrose in the medium.

These experiments were performed from June, 1951 to December, 1955. With each series of transfers, the stock cultures were checked for freedom from contaminating bacteria and fungi. Because the stock tissue used varied several days in age, this sometimes influenced the lag period required for the subcultures to begin active growth. The weights given in the results are those actually measured without correction to the weight of the control for a starting point.

Twenty-four tissue pieces were incubated on media in six bottles at each concentration in one experiment and each experiment was repeated three or more times. The tissues were harvested after 6 weeks incubation in the dark at 26° ± 1° C. Growth was measured as the average final wet weights of the tissues. The data in the curves are the averages of similar experiments made at different times. In all, over 18,000 tissue pieces were weighed. The statistical significance of the variability has already been studied (6).

Results

Both compounds and their concentrations influenced the amounts of growth of the callus cultures. The marigold and tobacco cultures sometimes grew at different rates. However, no macroscopic evidences were observed of differentiation by the tissues.

Growth with Purine Supplements: The influences on growth varied with the tobacco tissue when the cultures were incubated with different levels of the purines (fig 1). Growth increased slightly with the concentration of guanine and hypoxanthine. Growth with adenine and xanthine was not influenced until it was reduced at the highest concentration used.

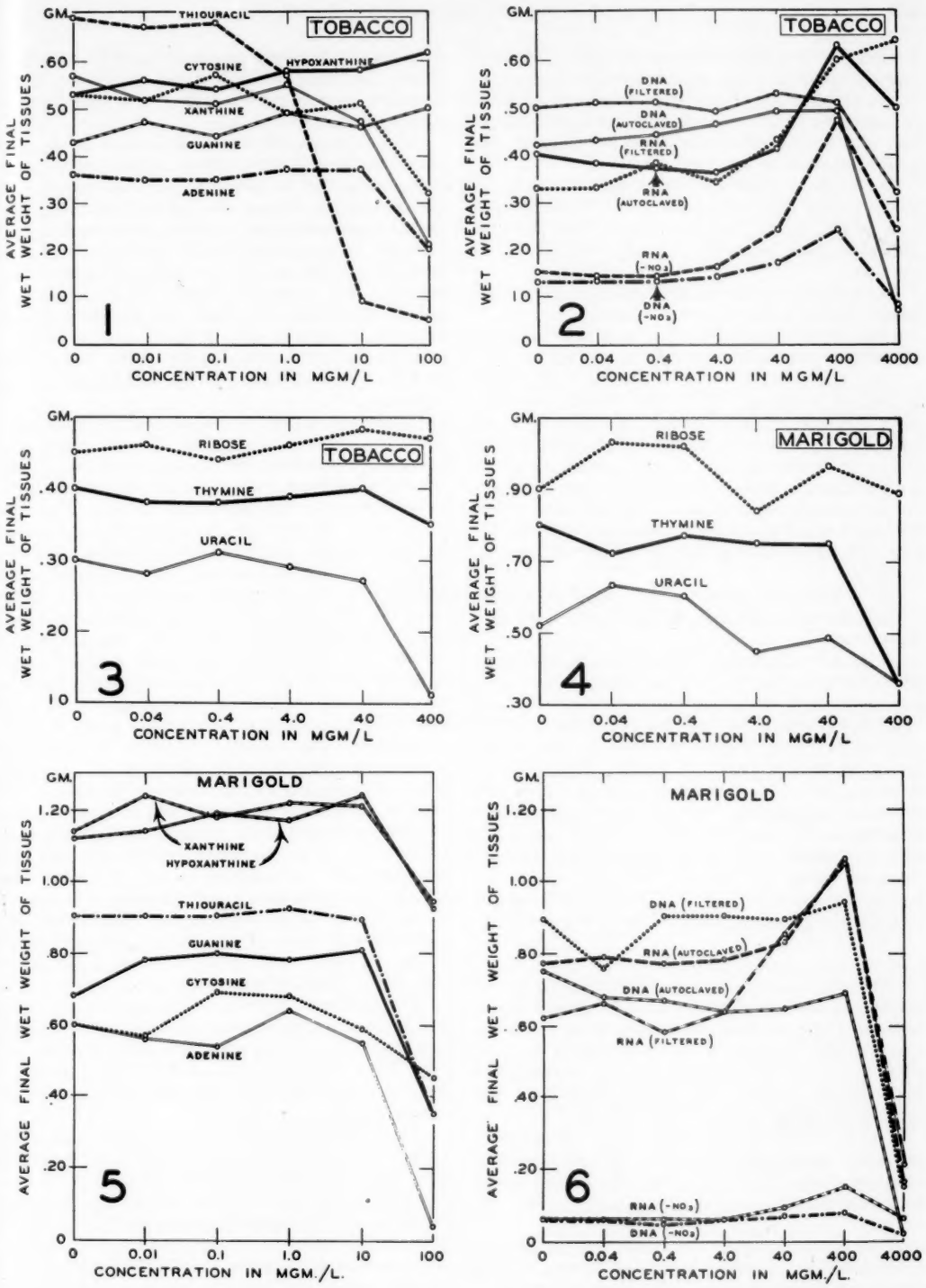
More favorable growth responses to the purines were displayed by the marigold tissues of crown gall origin (fig 5). With guanine, hypoxanthine, and xanthine, growth of this species increased slightly, perhaps, at all concentrations from 0.01 to 10 mg per liter. Adenine was beneficial, if at all, only at 1 mg per liter. All the purines inhibited growth to varying degrees at 100 mg per liter.

Growth with Pyrimidine Supplements: Cytosine and uracil influenced growth of tobacco cultures little, if any, until too strong (figs 1, 3). Thymine had little effect. Cytosine and particularly uracil inhibited growth at the highest concentration.

Marigold tissue appeared stimulated by certain dilutions of cytosine (fig 5) and uracil (fig 4). Thymine and uracil inhibited growth when strong enough.

Pentose Sugar: When D-ribose was added to the sucrose basal medium, little change was induced in the growth of tobacco or marigold tissue (figs 3, 4). Higher concentrations of D-ribose supported growth of tobacco comparable to the control media. However, the marigold and tobacco tissues failed to grow on media with any concentration of D-ribose as the sole source of carbon.

Ribonucleic Acid Supplements: The method of sterilizing RNA was important with tobacco tissue at 4 gm per liter—growth being better (fig 2) with the



FIGS. 1 to 6. Growth of marigold and tobacco tissue on media containing increasing concentrations, respectively, of nucleic acids, purines, pyrimidines, ribose or thiouracil. Each point on the curves represents the average wet wt after 6-wks incubation of 72 to 144 tissue pieces from experiments repeated 3 or more times.

filtered than with the autoclaved material. This tissue failed to grow at 8 and 16 gm per liter (not shown on the graph) with either filtered or autoclaved RNA. Marigold tissue was much benefited with 400 mg per liter of either autoclaved or filtered RNA (fig 6); but it was inhibited by 4 gm per liter.

As a source of nitrogen, RNA had little value for tobacco tissue below 40 mg per liter. It was satisfactory at 400 mg per liter, but not at 4 gm per liter. Marigold tissue grew little, if any, even at 400 mg per liter.

As a source of carbon, practically no growth appeared with 0.04 mg to 4 gm per liter.

The satisfactory growth by tobacco and the practical failure by marigold were striking at 4 gm per liter.

Deoxyribonucleic Acid Supplements: The method of sterilizing DNA also seemed important. With both tobacco and marigold tissues, growth was less (figs 2, 6) with autoclaved than with filtered DNA. In both cases, growth was satisfactory at 400 mg per liter, but was inhibited at 4 gm per liter.

As a source of nitrogen, DNA had little value, although tobacco tissue grew somewhat at 400 mg per liter. Marigold tissue grew little, if any, at any concentration.

As a source of carbon, DNA failed with both tobacco and marigold tissues at all of the usual concentrations.

Thiouracil Effects: Thiouracil along with some related compounds (11) was a highly active antimetabolite involved in inhibition of tobacco mosaic virus multiplication. It was also of interest to determine what, if any, effects this compound had on the growth in vitro of these two tissues. The results of three trials summarized in figures 1 and 5 showed that thiouracil supplements reduced growth of tobacco tissue some at a concentration of 1.0 mg/l, and strikingly by concentrations of 10 and 100 mg per liter. Marigold crown gall cultures were by comparison relatively tolerant of thiouracil. Only at 100 mg per liter was the growth of marigold tissue reduced 50 %.

Discussion

The tissue culture technique has provided an excellent tool for using well established bacteriological techniques to study fundamental physiology. For the interpretation of the results, considerations must be given to permeability, enzyme systems present, critical metabolic products from the substance tested, and variations between different plant species.

The concentrations that were effective might seem excessive when compared with that from an analysis of an entire plant. However, perhaps the amount should be compared with that within the cytoplasm and nucleus.

Furthermore, not only the normal but the diseased tissue deserved attention. In diseased tissues, whether they resulted from abnormal physiology or from some parasitic agent, the responses ranged from no effect to stimulation, injury or death. The tolerances which the cells had for common metabolic substances varied from species to species and doubtless were important. Such a tolerance by the host and intolerance by the pathogen have formed a basis for certain types of disease resistance.

The basic importance of the nucleic acids, their constituents, and their critical concentrations have been summarized by Chargaff and Davidson (2). In the plant tissue culture field, Miller and Skoog (12) described differentiation of tobacco stem segments into buds and leaves induced by the purines, adenine and guanine, and Skoog (18) has indicated that growth substance was intimately concerned with nucleic acid metabolism. Klein and Link (10) examined the importance of nucleic acids in crown gall and Nickell et al (15) in virus-infected tissue. Some of their findings have been extended and clarified by the present studies.

Thiouracil retarded tobacco mosaic virus multiplication in infected tissues (11, 17) and uracil suppressed the inhibition due to thiopyrimidines. That thiouracil inhibited virus multiplication at low levels offers promise for chemical control of diseased growth.

In the present studies, no differentiation occurred of callus cultures into buds, stems or roots. This was considered not at all contrary to the results of Miller and Skoog (12). Rather, it was due to basic differences between clones of tissue and procedures. Common observations have shown that freshly isolated tissues often differentiate into buds, roots or stems. However, such changes in established clones after long culture in vitro were less common than in fresh isolations. These cultures had been maintained as undifferentiated callus for over 14 years. Possibly the proper chemical balance was lacking; e.g., no additional indoleacetic acid was added to the culture medium in the present study and the endogenous levels of indoleacetic acid in the marigold and tobacco tissue cultures were not determined. Skoog (18) indicated the importance of indoleacetic acid in nucleic acid metabolism and in growth and cell division in tobacco stem segments. The conditions that might induce differentiation in these cultures were particularly interesting—especially what concentrations of the materials might affect differentiation.

The different responses of the cultures to RNA and DNA were conspicuous. Nickell et al (15) found this same phenomenon with virus-infected sorrel tissue. RNA favored growth of virus tumor tissue at low concentrations, but DNA was inhibiting at all concentrations.

These differential effects with DNA and RNA were of interest in connection with the crown gall initiation. For example, Klein and Link (10) suggested the importance of DNA in gall formation, since the DNA content increased in tomato plants 24 hours after inoculation with virulent crown gall bacteria. Klein and Klein (9) associated DNA isolated from virulent crown gall bacterial cultures with acquired virulence of avirulent crown gall bacterial cultures in media containing this source of DNA. In the present studies RNA was the stimulating material with in-

creased concentrations, while increased DNA concentrations had no stimulating effect. Both were inhibiting at higher concentrations.

The stimulating and inhibiting effects of RNA, depending on the concentration, were especially interesting in relation to symptoms with tobacco mosaic virus (TMV). Severely infected leaves commonly have shown both dark green areas that suggest growth stimulation and light green areas that suggest inhibition. Often parts of leaves failed to grow. This suggested complete inhibition. Holmes (7) and Fulton (3) have reported much less virus in the dark green portions than in the light green parts. The TMV concentrations in a composite sample of infected tissues averaged 1 to 2 mg per ml of sap. This average concentration of virus, which was mostly RNA, was between the amount of RNA that was stimulating to the tobacco tissue cultures and that which was inhibiting (figs 1 and 6). This elucidated the symptom formation in tobacco mosaic.

Summary

1. Tobacco tissue of normal origin from the hybrid *Nicotiana glauca* × *N. langsdorffii* (21) originally isolated and supplied by Philip White and marigold tissue of crown gall origin isolated in this laboratory were grown in chemically defined culture media with addition of nucleic acids, their components or the antimetabolite thiouracil.

2. Growth was strikingly stimulated by both filtered and autoclaved RNA at concentrations of 400 and 4000 mg per liter, while DNA had no effect at low concentrations and was strikingly inhibiting with 4000 mg per liter. RNA was furthermore an excellent source of nitrogen for the tobacco tissue but not for marigold tissue. DNA was not a good source of nitrogen for either tissue. Neither RNA nor DNA was a source of carbon for tobacco tissue.

3. Tobacco tissue was inhibited by 400 mg per liter of adenine, cytosine, xanthine, thymine, and uracil, while hypoxanthine, guanine and ribose supported progressively more growth with increasing concentrations.

4. Marigold cultures were inhibited by 400 mg/liter of all the purines and pyrimidines. Adenine and guanine were the most inhibiting. Some stimulation occurred with low concentrations of cytosine, guanine, and uracil and with ribose.

5. The antimetabolite thiouracil was strikingly inhibitory to tobacco cultures with concentrations of 10 or more mg per liter. Marigold cultures were inhibited by this compound only at 100 mg per liter.

6. The amounts of RNA that inhibited or stimulated these cultures, respectively, appeared comparable to the amounts inside stimulated dark green or inhibited light green tobacco leaves infected with TMV, which might clarify the characteristic mosaic symptoms.

The authors are indebted to Mr. Eugene Herrling for preparation of the illustrations.

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BRIEF PAPERS

THE ROLE OF CELL SAP CONCENTRATION IN FROST HARDINESS[1]

J. LEVITT

DEPARTMENT OF BOTANY, UNIVERSITY OF MISSOURI, COLUMBIA, MISSOURI

The many investigations attempting to relate cell sap concentration to frost hardiness have led to highly contradictory results (1). An indisputably close relation between the two exists in many cases; in many others there is a complete lack of correlation. A few investigators have even attempted to increase hardiness by inducing the uptake of relatively large amounts of solutes. The resulting increase in frost hardiness was always slight or nonexistent. The purpose of the present research is to reinvestigate the relation between frost hardiness and artificially induced increases in cell sap concentration, in an attempt to test the following hypothesis (1).

A primary hardiness factor establishes the amount of frost dehydration that will injure the plant. Cell sap concentration is a secondary factor; it increases hardiness by lowering the temperature at which this frost-killing dehydration will occur. But the extent of the increase is proportional to the frost dehydration that can be withstood without injury. If a plant is unable to survive ice formation at more than a few degrees below its freezing point, any increase in cell sap concentration will have a negligible effect on this frost killing point. But if it is able to survive a freeze of several degrees, an increase in cell sap concentration will appreciably lower this frost killing point. The quantitative relations are as follows:

If C_k = the cell sap concentration at the frost killing temperature, and C_f = the cell sap concentration at the freezing point, the ratio C_k/C_f represents the plant's tolerance of frost dehydration (1). As long as the primary hardiness factor is unchanged,

$$\frac{C_{k1}}{C_{f1}} = \frac{C_{k2}}{C_{f2}} = K \quad (1)$$

If, then, the cell sap concentration (C_f) is increased, C_k must increase proportionately. In other words, the frost killing point must be correspondingly lowered. Therefore, if C_{f_1} is increased to C_{f_2}:

$$C_{k2} = C_{k1} \times \frac{C_{f2}}{C_{f1}} \quad (2)$$

The frost killing point (T_k) must be correspondingly lowered from T_{k1} to T_{k2}. Knowing T_{k1}, C_{k1} can be obtained from a table of equivalent sucrose values (1). From the calculated value for C_{k2} (using equation (2) above), T_{k2} can be obtained using the same table.

The hypothesis can, therefore, be tested if the following values are determined:

1. The freezing point lowering (T_{f_1}) and frost-killing point (T_{k1}) for untreated plants. The corresponding cell sap concentrations (C_{f_1} and C_{k1}) can be obtained from a table of equivalent sucrose values.

2. The freezing point lowering (T_{f_2}) and frost killing point (T_{k2}) for corresponding plants whose cell sap concentrations have been artificially increased (to C_{f_2}) without altering the primary hardiness factor. By means of equation (2), cell sap concentration (C_{k2}) is calculated for the same amount of frost dehydration as in the untreated plant at C_{k1}. The calculated frost killing point (T_{k2}) corresponding to C_{k2} is obtained from the table of equivalent sucrose values.

If the hypothesis is correct, the experimentally determined and the calculated values for T_{k2} should agree.

METHODS

If, as postulated above, the effect of cell sap concentration on hardiness is proportional to the primary hardiness, appreciable differences will be obtained only when initially hardy plants are used. Consequently, in all the following experiments, the plants were hardened in a cold room at 3 to 5° C, (under a 200-watt light during the day) for 1 to 6 weeks before use. In order to increase the cell sap concentration without altering the primary hardiness factor, a relatively rapid uptake of a nontoxic and a non-metabolised solute is necessary. This was accomplished by infiltration at 5° C with glycerine solutions. Leaves were excised and immediately immersed in a solution inside a vacuum desiccator. They were covered by a wire screen to keep them below the surface of the liquid. The desiccator was then repeatedly evacuated and refilled with air until the leaves were completely infiltrated. To insure opening of the stomata, through which most of the solution enters, the leaves were excised 1.5 to 3 hrs after the light was turned on. In order to keep the stomata open during the infiltration process, a desk lamp was placed just above the desiccator.

Glycerine enters the cell reasonably rapidly; consequently, the infiltrated leaves were allowed to lose their intercellular water by transpiration (at 3 to 5° C) while this penetration occurred. All the intercellular water was lost in a few hours (cabbage) or 20 hrs (Saxifraga) if the leaves were simply laid flat on a wire screen 1 to 2 ft below the 200-watt bulb. If too high a concentration of glycerine was used for the rate of glycerine penetration to keep up with the rate of water loss by transpiration, collapse of some of the cells occurred. In the case of well hardened cabbage leaves such collapse was rare. The leaves were fully turgid and looked completely normal. When transferred to the laboratory and placed in water, they remained alive for days or even weeks,

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TABLE I

AMOUNT OF MOLAR GLYCERINE INFILTRATED INTO HARDENED CABBAGE LEAVES

LEAF NO. (FROM BASE UP)	LEAF WT, GM		UPTAKE OF M GLYCERINE	
	BEFORE INFILTRATION	AFTER INFILTRATION	WT, GM	% OF LEAF WT
		Plant no. 1		
10	2.60	3.15	0.55	21.2
11	2.35	2.85	0.50	21.3
12	2.55	3.05	0.50	19.6
				Av 20.7
		Plant no. 2		
11	1.00	1.15	0.15	15.0
12	0.90	1.05	0.15	16.7
13	1.45	1.70	0.25	17.2
				Av 16.3

and were completely indistinguishable from untreated controls. A few whole cabbage plants were also infiltrated by inverting pots in beakers of glycerine solutions under a bell jar. Again, no sign of injury occurred as long as the glycerine solution was not too concentrated to permit loss of intercellular water without cell collapse. The whole process of infiltration and loss of intercellular water required only 6 to 12 hrs in the case of cabbage, 24 hrs in the case of Saxifraga. In such short periods of time (all at 5° C), no appreciable change in the primary hardiness factor would be expected.

Cell sap concentrations were determined plasmolytically from the concentration of $CaCl_2$ just sufficient to cause incipient plasmolysis. Since this reveals actual changes in the living cell, it is preferable to freezing point determinations which would give only an average value—including the glycerine in the non-living vascular tissues, etc. In order to reduce to a minimum the loss of glycerine to the plasmolysing solution, the determinatoins were made as quickly as possible—within about fifteen minutes.

As a rough check of the plasmolytic method, the amount of glycerine solution taken up by the leaves was determined by weighing before and after infiltration (table I). From these values it was possible to calculate the increase in cell sap concentration when all the glycerine had penetrated the cells. These calculated values agreed as well as can be expected with the plasmolytically determined values (table II). Similar checks with Saxifraga gave even closer agreement. Consequently, no serious error can have occurred due to loss of glycerine from the cells during the plasmolytic determinations. As a further check, $CaCl_2$ solutions containing the same partial pressure of glycerine as was calculated to be inside the cells gave the same osmotic potentials at incipient plasmolysis as did the pure $CaCl_2$ solutions.

TABLE II

INCREASE IN CELL SAP CONCENTRATION OF HARDENED CABBAGE LEAVES DUE TO INFILTRATION WITH MOLAR GLYCERINE

LEAF NO.	TIMES INFILTRATED	OSMOTIC POTENTIAL, ATMS		CELL SAP CONC, ATMS	
		BEFORE INFILTRATION	AFTER INFILTRATION	MEASURED INCREASE	CALCULATED INCREASE (FROM TABLE I)
1	Once	19.9	25.2	5.3	4.8– 6.0
	Twice	...	28.7	8.8	9.6–12.0
2 (slightly hardened)	Once	14.3	20.6	6.3	4.8– 6.0
3	Twice	20.3	28.7	8.4	9.6–12.0
4	Once	21.9	25.2	3.3	4.8– 6.0

TABLE III

EFFECT OF INFILTRATION WITH MOLAR GLYCERINE ON FROST KILLING TEMPERATURE (T_k) OF CABBAGE LEAVES

TIME HARDENED, WKS	TREATMENT	OSMOTIC POTENTIAL, ATMS	C_{t_2}/C_{t_1}	T_k, °C	
				DETERMINED	CALCULATED
1.5	Control	20.25	...	– 7	...
	Infiltrated twice	27.0	1.34	–11	–10
2.5 (whole plant)	Control	21.9	...	– 7	...
	Infiltrated once	25.2	1.15	– 9.5	– 8.5
8	Control	25.2	...	–12.5	...
	Infiltrated once	32.3	1.25	–15.5	–15.5

Frost hardiness was determined as follows. Both infiltrated and control leaves were suspended in a thermostatically controlled deep freeze at about – 5° C by inserting their labels into paper clips hanging from a string running from wall to wall. A thermometer was also suspended so that the bulb was at the same level and close to the leaves, and a thermograph just below them recorded the temperature. The cut surface of each petiole was immediately inoculated with snow to prevent undercooling. The leaves were always frozen solid within an hour. The temperature

TABLE IV

EFFECT OF INFILTRATION WITH MOLAR GLYCERINE ON FROST KILLING TEMPERATURE (T_k) OF SAXIFRAGA CORDIFOLIA LEAVES

TIME HARDENED, WKS	TREATMENT	OSMOTIC POTENTIAL, ATMS	C_{t_2}/C_{t_1}	T_k, °C	
				DETERMINED	CALCULATED
6	Control	21.9	1.28	–16	...
	Infiltrated	28.7	...	–20	20.5
7	Control	18.6	1.5	–15	...
	Infiltrated	28.7	...	–17.5	–22.5
8	Control	18.6	1.5	–13.5	...
	Infiltrated	28.7	...	–17.5	–20

was then dropped at the rate of not more than 1° per hour. The leaves were kept at the desired freezing temperature long enough to reach equilibrium (2 to 3 hrs). Two treated and two control leaves were then removed to the cold-room (3 to 5° C), the others were left in the chamber and the temperature dropped another one or two degrees. A second set was removed to the cold room and the temperature again dropped. In this way a range of temperatures was used. After 24 hours for recovery in the cold room, the leaves were transferred to the laboratory and kept with their petioles in water for a week, after which the injury was estimated. A series of percentage injuries (e.g., 0, 30, 70, 100 %) enabled determination of the frost killing temperature—the temperature causing 50 % killing. In order to obtain as uniform leaves as possible, leaves from the same nodes on similar plants were always compared.

Results

In contrast to the results of earlier investigators (see 1), increasing the cell sap concentration always increased the frost hardiness (tables III and IV and fig 1). This can be explained by the fact that for the first time the plants used were already frost hardy. In all cases, the calculated values for the frost killing temperatures of the infiltrated cabbage leaves agreed with the experimentally determined values, within the limits of error of the method (table III). This was true for all degrees of hardening used, and whether excised leaves or whole plants were compared. In the case of Saxifraga, the calculated values were apparently slightly lower than the determined (table IV).

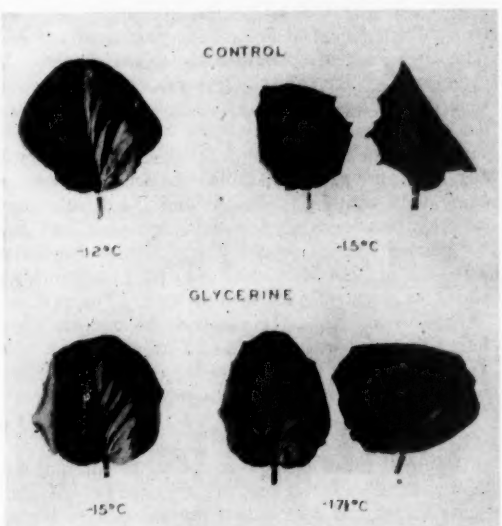

Fig. 1. Relative frost injury to control and glycerine-treated hardened leaves of *Saxifraga cordifolia*. No injury to control at −12° C and to glycerine-treated leaves at −15° C, 50 and 100 % injury to controls at −15° C, 25 and 50 % injury to glycerine-treated leaves at −17° C.

The importance of these results is twofold:

1. The osmotic effect of the solute completely and quantitatively accounts for the increase in frost hardiness. There is no place for any other effect, such as a protective one on the protoplasmic proteins. This method should then be applicable to test the role of sugars in hardiness.

2. The increased solute content lowers the temperature survived and therefore the degree of dehydration (i.e., the vapor pressure) tolerated. But the calculated amount of ice formed is the same; therefore, it does not alter the amount of dehydration tolerated—i.e., the tolerance of frost dehydration. These then are two distinct factors in frost hardiness.

The slight discrepancy in the case of Saxifraga can be explained in two ways. (a) It must be remembered that at the lower temperatures required to kill these plants, the values for the sucrose solutions from which the theoretical frost killing points are derived are based on extrapolation and not on experiment (Levitt, 1956). But this error would be expected to be in the opposite direction—i.e., the extrapolated values are probably not low enough. (b) The cells' tolerance of dehydration cannot be completely independent of temperature. The lower the temperature, the stiffer the protoplasm must become (even if the hydration is constant) and the more readily it can be injured mechanically. Consequently, we can expect a slight decrease in tolerance of dehydration with drop in temperature, particularly at the lower temperatures since the dehydration is more severe.

Summary

1. Hardy plants of both cabbage and *Saxifraga cordifolia* showed very definite increases in frost hardiness (2 to 6° C) as a result of infiltration with molar glycerine solutions, followed by evaporation of the water so absorbed.

2. In the case of cabbage, the increases in hardiness agreed very well with the calculated increases on the basis of a purely osmotic effect. In the case of Saxifraga, the increases were somewhat less than expected.

3. It is suggested that the results with Saxifraga are related to the lower temperatures that this plant can survive. The increased stiffening of the protoplasm with the drop in temperature may partly counteract the osmotic protection by slightly reducing the tolerance of dehydration.

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ALTERING THE PIGMENTS PRODUCED BY TYROSINASE AND ORTHO-HYDROXYPHENOLS WITH A META-HYDROXY-PHENOL, 4-CHLORORESORCINOL[1]

PAUL E. WAGGONER AND A. E. DIMOND

DEPARTMENTS OF SOILS AND CLIMATOLOGY AND OF PLANT PATHOLOGY AND BOTANY,
CONNECTICUT AGRICULTURAL EXPERIMENT STATION, NEW HAVEN, CONNECTICUT

Tyrosinase oxidizes *o*-hydroxyphenols to form pigments. Substances such as chelating agents and structural analogues of *o*-hydroxyphenols (2) can prevent the formation of pigment as inhibitors. Other compounds such as β-keto (6) and amino acids (4) alter the color produced. The latter are of interest because they are not inhibitors in a strict sense and because in certain biological reactions, as in the production of brown pigment in the curing of tobacco leaves, a control of the quality as well as the quantity of color is desirable. Therefore, a knowledge of the kinds of compounds that can alter the quality and of how these compounds produce their effects is useful from a practical as well as theoretical point of view.

Such knowledge could lead to an understanding of how a wide variation in color arises in the discoloration of woody tissues caused by fungi in plant diseases and related problems. The brown vascular discoloration which is a prominent symptom of Fusarium wilt of tomato is probably a result of tyrosinase activity (1). Vascular discoloration by other wilt pathogens may occur by similar mechanisms. If other compounds affect the quality of pigment formed by tyrosinase acting upon its natural substrate, one may be able to answer certain questions. Why does *Verticillium albo-atrum* produce brown pigments when it attacks solanaceous hosts but green ones when it attacks maple? Why does *Ceratostomella ulmi* produce brown discoloration in elm while *C. ips* produces a blue stain in lumber? Perhaps the cause of discoloration in all such cases is oxidation of *o*-hydroxyphenols by oxidase and the presence of small amounts of other compounds causes color to be altered from brown to red, green, or blue.

The present studies were begun when the *m*-hydroxyphenol, 4-chlororesorcinol, when added to tyrosinase plus an *o*-hydroxyphenol was found to produce a red rather than a brown color. 4-Chlororesorcinol is known to prevent pigment formation from 3,4-dihydroxyphenylalanine and catechol (3, 7). But when tyrosinase acts on chlorogenic acid, the addition of 4-chlororesorcinol changes the color of the pigment produced. Thus alteration of the color, and presumably the pigment, depends not only upon the enzyme but upon the substrate as well. Our experiments suggests how these events occur with known substrates and with ones that occur naturally.

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MATERIALS AND METHODS

Thick peels of washed Katahdin potato tubers were used as a source of tyrosinase. The peels were chilled to 0° C and homogenized in 3 ml of 0.1 M pH 7 sodium phosphate per gm wet weight of tissue and centrifuged. The clear supernatant fluid was dialyzed against distilled water. This preparation was free from peroxide as shown by its failure to oxidize hydroquinone without added H_2O_2. Chromatographically pure chlorogenic acid was furnished by General Foods, Inc. Catechol was purified by sublimation. 3,4-Dihydroxyphenylalanine and 4-chlororesorcinol were Eastman white label grade.

The reactions of known substrates and 4-chlororesorcinol were followed by chromatographing sequential, 0.05-ml aliquots of the solutions. The reactions were stopped by drying. Ascending chromatograms on No. 3 Whatman paper were developed overnight in the organic phase of butanol - acetic acid - water (4:1:5). The chromatograms were examined under visible and ultraviolet (254 mμ maximum) light and sprayed with modified Folin-Denis reagent (5) or with ninhydrin reagent. Compounds were identified by color and by R_f values. The relative quantities of each compound were estimated by ranking in the order of density of color.

Darkening due to naturally occurring substrates, enzymes, and pH was studied in potato tubers because they contain tyrosinase and the 2 substrates, 3,4-dihydroxyphenylalanine and chlorogenic acid, with no pigment which would obscure color formation. Two ml of 0.01 M 4-chlororesorcinol solution was added to 2 gm of peel in a mortar. The peel was bruised to initiate darkening and the mixture transferred to a vial with the aid of 2 ml of water. After 24 hours at room temperature the fluid was decanted, centrifuged, and examined in a spectrophotometer.

RESULTS

One-half ml of enzyme in 1.5 ml of solution containing 1 millimolar chlorogenic acid and 0.07 M phosphate buffer, pH 6.3, caused the solution to become brown. If 1 millimolar 4-chlororesorcinol was also present, the solution became red. If either the enzyme or chlorogenic acid was omitted, no color change occurred. The rate of these changes was followed chromatographically (table I). As expected, the brown pigment at $R_f = 0$ arose from oxidation of chlo-

rogenic acid. A pigment with $R_f = 0.7$ appeared on the chromatograms as a red spot under ultraviolet light when the red solutions were chromatographed. This pigment apparently arose from both 4-chlororesorcinol and chlorogenic acid, but less chlorogenic acid was oxidized than when 4-chlororesorcinol was absent.

The pigment changed rapidly during the first few minutes. It was not feasible to stop the reaction by boiling because of the sensitivity of the red pigment to heat. For this reason the absorption spectrum after 11 minutes of reaction was approximated as follows: spectrographic observations of the pigment formed from 0.33 millimolar chlorogenic acid and 0.33 millimolar 4-chlororesorcinol by 0.1 ml of crude enzyme in 3 ml of solution were begun after 6 minutes. The first observation was made at a wave-length of 400 mμ. Then observations were taken as rapidly as possible at increasing wavelength until 600 mμ was reached. The order of reading was next reversed and 400 mμ was reached after 16 minutes of reaction. The 2 readings taken at each wavelength were averaged to obtain the spectrum at 11 minutes. The spectrum revealed a plateau from 400 to 450 mμ and a rounded peak at 490 mμ. As time passed, the optical density at 400 to 430 mμ rose while that at 490 mμ showed little change. Thus, the initial, brilliant red color of the solution became more brown. Hence, the ratio of the density at 500 mμ to that at 400 mμ provides a measure of the amount of red pigment relative to the amount of brown pigment.

The optimal concentration for formation of red pigment from chlorogenic acid and 4-chlororesorcinol was determined (table II). High ratios of optical densities at 500 mμ to those at 400 mμ show that the highest relative concentrations of red pigment were produced with high and equal concentrations of substrate and 4-chlororesorcinol.

TABLE I

PIGMENTS FORMED FROM CHLOROGENIC ACID AND 4-CHLORORESORCINOL RANKED FOR EACH SUBSTANCE ACCORDING TO DENSITY OF COLOR ON A CHROMATOGRAM

		R_f			
REACTION TIME	4-CHLORORESORCINOL	0.95 4-CHLORORESORCINOL	0.70 RED FLUORESCENT PIGMENT	0.65 CHLOROGENIC ACID	0 BROWN PIGMENT
min	*mM*		*Ranks* *		
0	0	0	0	4.5	2
0	1	4	0	4.5	2
1	0	0	0	1	5.5
1	1	3	0	3	2
10	0	0	0	0	7
10	1	2	0	2	4
100	0	0	0	0	8
100	1	1	1	0	5.5

The reaction mixture contained 0.5 ml crude enzyme, 1 millimolar chlorogenic acid, and 0 or 1 millimolar 4-chlororesorcinol in 0.07 M, pH 6.3, sodium phosphate, with a final volume of 1.5 ml.

* Ranks assigned from zero for none to the highest number for the most dense color for the compounds observed on the chromatograms.

TABLE II

RATIO OF OPTICAL DENSITY OF SOLUTION AT 500 MΜ AFTER 3 MINUTES TO THAT AT 400 MΜ AFTER 2 MINUTES

CHLOROGENIC ACID, MM	4-CHLORORESORCINOL, MM			
	3.3	0.33	0.033	0
3.3	1.2	0.66	0.25	0.16
0.33	0.75	1.2	0.71	0.17
0.033	0.52	0.78	0.72	0.14
0		No color		

The reaction mixture contained 0.1 ml crude enzyme in 0.07 M, pH 6.3, sodium phosphate.

A test was made of the effect of 4-chlororesorcinol upon the formation of pigment from 3,4-dihydroxyphenylalanine. The methods were the same as for the experiment summarized in table I with 1 millimolar 3,4-dihydroxyphenylalanine replacing chlorogenic acid. 3,4-Dihydroxyphenylalanine disappeared rapidly in the presence of the enzyme, and a dark pigment appeared. However, when 4-chlororesorcinol had been added, no decrease in either 4-chlororesorcinol or substrate could be seen after one hour. No new pigment was formed.

4-Chlororesorcinol also influenced the action of tyrosinase upon its classical substrate, catechol. In a similar experiment, 4-chlororesorcinol delayed the disappearance of catechol and the appearance of brown pigment. A new, green pigment appeared at $R_f = 0.03$. However, the concomitant decrease in substrate and 4-chlororesorcinol as shown by a chromatogram was too small to reveal the source of the new pigment.

The color changes with naturally occurring substrates and enzymes, i.e., in potato peels, were predicted from the results of the preceding experiments with known substrates. The production of red pigment above required equal amounts of substrate and 4-chlororesorcinol. The 2-gm fresh weight of peel used contained 2 to 4 micromoles of chlorogenic acid (5). Twenty micromoles of 4-chlororesorcinol in the solution were expected to diffuse into the site of reaction in the peel to the extent of 2 to 4 micromoles and cause a red color to appear rather than the brown one produced in untreated peel. This was expected because the normal conversion of chlorogenic acid into brown pigment would be diverted to the formation of a red one and the normal conversion of 3,4-dihydroxyphenylalanine into blue pigment would be inhibited.

Untreated, crushed peels produced a brown, water-soluble pigment in 24 hours. The absorption spectrum of the centrifuged solution showed a smooth, rapid decrease of density at increasing wavelengths in the visible range. Crushed peels treated with 4-chlororesorcinol produced a pink, water-soluble pigment in 24 hours. The absorption spectrum of this solution showed a plateau from 435 to 490 mμ. Further, the optical densities of the treated sample were less in the 400–500 mμ region than those of the untreated

one showing that the quantity of pigment had been decreased.

Crushed peels which had stood for 96 hours were macerated by bacteria, undoubtedly *Erwinia carotovora*. The untreated peels became black, while the peels treated with 4-chlororesorcinal remained pink.

Discussion

The color as well as the quantity of the pigment formed by tyrosinase was controlled by 4-chlororesorcinol. Whether the quantity was decreased by inhibition or whether an entirely new pigment was formed depended upon the substrate and its concentration. In the case of 3,4-dihydroxyphenylalanine, Kull et al (7) have already shown that 4-chlororesorcinol inhibits the formation of pigment. In the case of catechol, Heymann et al (3) have shown that low concentrations of 4-chlororesorcinol inhibit oxidation and a transitory, red pigment appears; in our experiments a green pigment appeared when higher concentrations of the inhibitor were used. In the final case, our results show that a red pigment appears when chlorogenic acid is acted upon by tyrosinase in the presence of an equal concentration of the inhibitor. Thus, 4-chlororesorcinol is valuble for demonstrating the action of tyrosinase in a naturally occurring system as was shown in the experiment with potato peel.

The effect of 4-chlororesorcinol in decreasing darkening and causing the formation of a new pigment may have an application in the processing of such things as tobacco, tea, and coffee. The results will depend upon the identity and amount of polyphenols present. For example, M. Zucker (unpublished research) has found that the addition of 4-chlororesorcinol to tobacco leaf homogenates causes a red rather than a brown pigment to be produced.

A symptom of disease may be incidental to or a major cause of injury to the host. In the case of the potato tuber decay, 4-chlororesorcinol prevented blackening without interrupting the major series of events that led to offensive odor and rotting of tubers. We conclude that blackening is a side event that need not occur before the host can be critically injured. The same would be true in wilt diseases such as Fusarium wilt of tomato if the discoloration can be prevented and yet the plant wilts and dies. On the other hand, in a fungal damage such as blue stain of wood products the discoloration is the major damage; here prevention of discoloration would prevent any loss.

Summary

4-Chlororesorcinol forms a red pigment instead of the usual brown one which is produced when tyrosinase acts upon chlorogenic acid. The disappearance of chlorogenic acid was slower than 4-chlororesorcinol was added, but both compounds were consumed as the red pigment was formed. The red pigment was produced in highest relative concentrations when equivalent amounts of substrate and the *m*-hydroxyphenol were present. Darkening of potato tuber peels due to crushing or decay was decreased by 4-chlororesorcinol and pink pigment was produced instead. The change in pigments has implications and applications in plant processing and disease.

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THE EFFECT OF 2,4-DICHLOROPHENOXYACETIC ACID ON THE ACCUMULATION OF MINERAL ELEMENTS IN TOBACCO PLANTS[1,2]

CARRICK E. WILDON, CHARLES L. HAMNER AND SAMUEL T. BASS

DEPARTMENTS OF HORTICULTURE AND AGRICULTURAL CHEMISTRY, MICHIGAN STATE UNIVERSITY, EAST LANSING, MICHIGAN

Since the discovery of the herbicidal action of growth regulators such as 2,4-dichlorophenoxyacetic acid (2,4-D), there has been much information obtained concerning the herbicidal mechanism. The action of 2,4-D on organization, physiology and chemical changes of cells and tissues has been studied (6, 7, 8, 9, 10). Several experimenters (2, 5, 6, 9) on different plants have found that 2,4-D induces rapid depletion of carbohydrate reserves. Some information has been presented on accumulation of mineral elements in plant parts as affected by applications of growth regulators at higher concentrations (5). Less information is available concerning the action of growth regulators on mineral uptake of plants and such information is somewhat contradictory (7, 8, 10). Additional investigation is needed on the effect of soil applications of 2,4-D in herbicidal concentrations on the mineral intake of a vigorously growing plant.

Wolf et al (10) reported that higher levels of nitrate in the substrate increased the sensitivity of soybeans to toxic concentrations of 2,4-D. Rhodes, Templeman and Thruston (9) found that rape treated with 2-methyl, 4-chlorophenoxyacetic acid (MCPA) failed to accumulate potassium in the tops altho there was accumulation in tops of untreated plants. There were no observable effects on phosphorus intake. Tomato plants treated with MCPA, accumulated potassium in the roots but not in the tops. The total intake by the entire plant was reduced. Similar but less obvious trends in accumulation of phosphorus and total nitrogen were also noted in tomato plant treated with MCPA. No influence on calcium and magnesium content of treated tomato plants was noted. Wolf et al (10) found that soybeans grown on a high nitrate substrate took up less sulfur and phosphorus when the roots were treated with 2,4-D. They also found potassium content lower and calcium content higher in leaves of treated plants, altho when stem and leaves were combined, differences largely disappeared.

Loustalot et al (4) have shown an increase of phosphorus content of white bean plants treated with 2,4-D. Rebstock et al (7) noted that total phosphorus in cranberry bean plants treated with 2,4-D was less than in non-treated plants.

Young seedlings of tobacco var. Common Havana were selected for uniformity and planted into eight-inch pots of sand to which had been added a complete fertilizer. Dilutions of major nutrient mineral elements were applied to the soil of each plant weekly to maintain near maximum growth. The plants were grown to a height of twelve inches and then arranged in two groups of ten each.

Since Wolf et al (10) had reported that sensitivity to 2,4-D is closely correlated with nitrogen nutrition, each plant received an equivalent amount of a solution of ammonium sulfate and potassium nitrate poured into the sand around the plant three days previous to treatment. Each of five plants of each group were given 50 ml of a 0.1 % solution of sodium salt of 2,4-dichlorophenoxyacetic acid poured into the sand around the plant. The remaining five plants of each group were used as controls and received no 2,4-D. No additional nutrients were added after treatment with 2,4-D. The plants were harvested two weeks after treatment, dried and analyzed for mineral content. Sodium and potassium were determined by flame photometric methods (1). Remaining elements were determined by spectro-chemical methods (Bacon, R. A. and Bass, S. T., unpublished).

TABLE I

MINERAL CONTENT OF TOBACCO PLANTS AS AFFECTED BY 2,4-DICHLOROPHENOXYACETIC ACID

ELEMENTS	TREATED REPLICATES		UNTREATED REPLICATES	
	TOP	ROOT	TOP	ROOT
	% dry wt			
Boron	0.0028	0.0123	0.0017	0.0103
	0.0022	0.0126	0.0018	0.0114
Calcium	2.60	3.27	2.47	2.93
	2.33	4.02	2.53	1.80
Copper	0.0057	0.0096	0.0030	0.0178
	0.0014	0.0061	0.0016	0.0193
Iron	0.0530	0.7116	0.0302	0.6143
	0.0357	0.8252	0.0279	0.7218
Magnesium	0.791	1.34	0.769	1.37
	0.715	1.36	0.852	0.963
Manganese	0.0070	0.0185	0.0079	0.0185
	0.0062	0.0199	0.0079	0.0200
Potassium	1.94	1.81	2.57	1.79
	1.76	1.86	2.97	1.48
Phosphorus	0.167	0.303	0.259	0.326
	0.159	0.363	0.318	0.371
Sodium	0.029	0.097	0.061	0.106
	0.035	0.083	0.061	0.143
Zinc	0.0074	0.0099	0.0065	0.0064
	0.0056	0.0055	0.0066	0.0052

[1] Received January 22, 1957.

[2] Michigan Agricultural Experiment Station Journal Article 2009.

Plant tops of treated plants contained a lesser percentage of potassium, sodium, and phosphorus and a greater percentage of boron and iron than did the untreated plants, table I. Roots of treated plants accumulated more calcium and less copper than roots of untreated plants. There was little or no difference in accumulation of calcium, copper, magnesium and zinc in tops of the plants nor in accumulation of boron, potassium, magnesium, manganese, and phosphorus in roots of the plants as between treated and untreated plants, table I.

Plant roots contained greater percentages of boron, copper, iron, magnesium, manganese, sodium, and phosphorus than plant tops in both treated and untreated plants. Calcium accumulated to a greater amount in roots than in tops of treated plants, altho no differences were noted in untreated plants. Potassium accumulated to a greater extent in tops than in roots of untreated plants, while no differences were noted in treated plants.

The content of phosphorus in the tops of the treated plants is much less than that of untreated plants. Grizzard et al (3) has demonstrated that rate of growth of tobacco parallels the rate of absorption of phosphorus.

The content of potassium in the tops of treated plants was 2/3 that of untreated plants.

Summary

Information on the action of growth regulators on mineral uptake of plants is rather scarce and somewhat contradictory.

Fifty ml of a 0.1 % solution of sodium salt of 2,4-D was poured into the sand around young tobacco plants grown in sand in eight-inch pots. Two weeks later plants were harvested, dried and analysed for mineral elements.

Content of phosphorus and potassium in the tops of treated plants was much less than in the tops of untreated plants.

Calcium accumulated to greater amount in roots than in tops of treated plants, but there was no difference in accumulation of calcium in roots and tops of untreated plants.

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A DARKROOM SAFELIGHT FOR RESEARCH IN PLANT PHYSIOLOGY[1,2]

R. B. WITHROW and L. PRICE
Smithsonian Institution, Washington, D. C.

The selection of a suitable safelight system for handling plant material in the darkroom always has been a difficult problem. This is especially true for studies of chlorophyll synthesis and the photocontrol reactions of phototropism and photomorphogenesis, where measurable plant responses are obtained at very low irradiances near the threshold of vision. Most investigators of phototropism and auxin physiology have used a red safelight which emits energy in the general region of from 600 mμ out into the infrared, since the phototropic action spectrum has its maximum in the blue and negligible response in the orange, red and infrared, and because the classical organ for such studies, the Avena coleoptile, requires a pre-exposure to red radiant energy for suppression of the mesocotyl.

This practice is open to serious question in view of the observations by Kent and Gortner (5) that, in the pea test, little curvature in response to alpha-naphthalene acetic acid was secured without a pre-exposure to red radiant energy. Klein, Withrow and

[1] Received January 22, 1957.
[2] Published with the approval of the Secretary of the Smithsonian Institution.

Elstad (6) found that the concentration of externally applied auxin required to produce a specified angle of the hypocotyl hook of bean was dependent on the dose of red energy. Conversely, it was demonstrated that the radiant energy required to produce a given hook-opening response was related to the external supply of auxin, i.e., the higher the level of auxin, the higher the incident energy required. Plant materials to be used in studies of this type usually are exposed to a safelight for varied and unmeasured periods of time depending upon the speed with which the operator can carry out the manipulations. This introduces an unknown variable which may condition the response.

Within the past five or six years, quite a few investigators have used green safelights, including Koski et al (7) for chlorophyll synthesis and Curry et al (3) for phototropism. However, the spectral characteristics of the system have usually not been specified. Nitsch and Nitsch (8) employed a green safelight devised by C. Yocum. This consisted of a green fluorescent lamp wrapped in three layers of "amber" and "green" cellulose acetate. Dr. Yocum (private communication, February, 1957) has indicated that little phototropic curvature resulted when Avena coleoptiles were exposed two inches from the source for one hour. He states that some red is visible with this source and, therefore, it would not appear to be satisfactory for studies involving the red, far-red group of reactions.

To be adaptable for a range of photobiological investigations, a safelight should have its maximum transmission near the region of maximal spectral sensitivity of the human eye and should exclude those wavelengths inducing phototropism, chlorophyll synthesis or photomorphogenesis. Weaver (10) and others have shown that the dark-adapted eye has maximum sensitivity at 510 mμ while the light-adapted eye, i.e., one which has been exposed to relatively high light intensities, has its maximum sensitivity at 555 mμ. When working under the lowest possible light intensities, the dark-adapted eye is color blind and makes use of rod vision only. Its spectral sensitivity is given in figure 1. It will be noted that the sensitivity in the green at 510 mμ is from 100 to 1000 times greater than in the orange-red. For the same visibility, it is possible to use a very much lower irradiance in the green than in the orange and red.

The processing of photographic material presents a problem similar to that of handling photosensitive plant materials. With orthochromatic emulsions, which have little orange and red sensitivity, an orange safelight is used. Panchromatic materials are sensitive throughout the entire visible spectrum and it is essential to use a safelight which yields maximum visibility per unit of transmitted energy. In this case, a green safelight is recommended. The green Wratten-type safelight filters designed for use in handling film, however, transmit in the far-red. The commercial safelight filters which have been tested in this laboratory begin to transmit just beyond 720 mμ. They are not, therefore, very suitable for handling plant material to be used in photomorphogenic studies, especially with incandescent sources, since photoinactivation of this process has its maximum activity in the general region of from 700 to 750 mμ (1, 12, 13).

The curves of figure 1 present the action spectra for the principal plant photochemical reactions, together with the spectral sensitivity of the dark-adapted eye taken from the data of Weaver (10). It will be noted that the phototropic action spectrum (4) falls to a minimum at about 520 mμ, while the maximum sensitivity of the dark-adapted eye is at 510 mμ. In the region of 500 to 550 mμ, there is a general low level of sensitivity for chlorophyll synthesis (7), photosynthesis (2), and photomorphogenesis (6, 12, 13).

The safelight we have devised for our work consists of two General Electric green fluorescent lamps filtered with dyed-gelatin films which we prepare in our laboratories according to the methods described previously (11). This safelight has been mentioned in a previous publication (6), but it has given us such excellent service for a variety of research problems that we feel it merits a more detailed description. The first filter (A, table I) we prepared was very satisfactory for red photomorphogenic studies but a weak bending response in Avena coleoptiles was excited. This filter was modified by adding more of the yellow dye and less of the green, so as to shift the band about 10 mμ to the longer wavelengths. These filters have been used for over six years in studies of photomorphogenesis and about one for phototropism. Transmission is from 500 to 550 mμ for filter A, and 510 to 580 mμ for filter B. The shaded curve of figure 1 gives the calculated relative spectral distribution of the combination of a green fluorescent lamp and two films of filter A. From figure 2 it can be seen that the green fluorescent lamp is a much more suitable safelight source than the incandescent lamp, especially for investigations involving the photomorphogenic red and far-red reactions. The incandescent lamp has its maximum energy in the infrared; this would be of little consequence except that organic dyes transmit freely in the near infrared and most of them in the far-red. With the green fluorescent lamp, the problem is minimized because there is little energy in the longer wavelengths except for that from the incandescent cathodes.

The transmittances of two safelight filters are given in table I, together with the formulas for their preparation. The filters do not begin to transmit in the infrared until about 800 mμ. This has been achieved by adding high concentrations of naphthol green B. Since the green transmission band of naphthol green B is broad, tartrazine and pontacyl green BL were added to narrow the band to the desired limits. The combination of the three dyes is dissolved in a solution of gelatin, sorbitol and sodium benzoate at the rate of 0.3 mg of each dye per cm^2 of gelatin-film surface. One film of filter A of 1000 cm^2 area

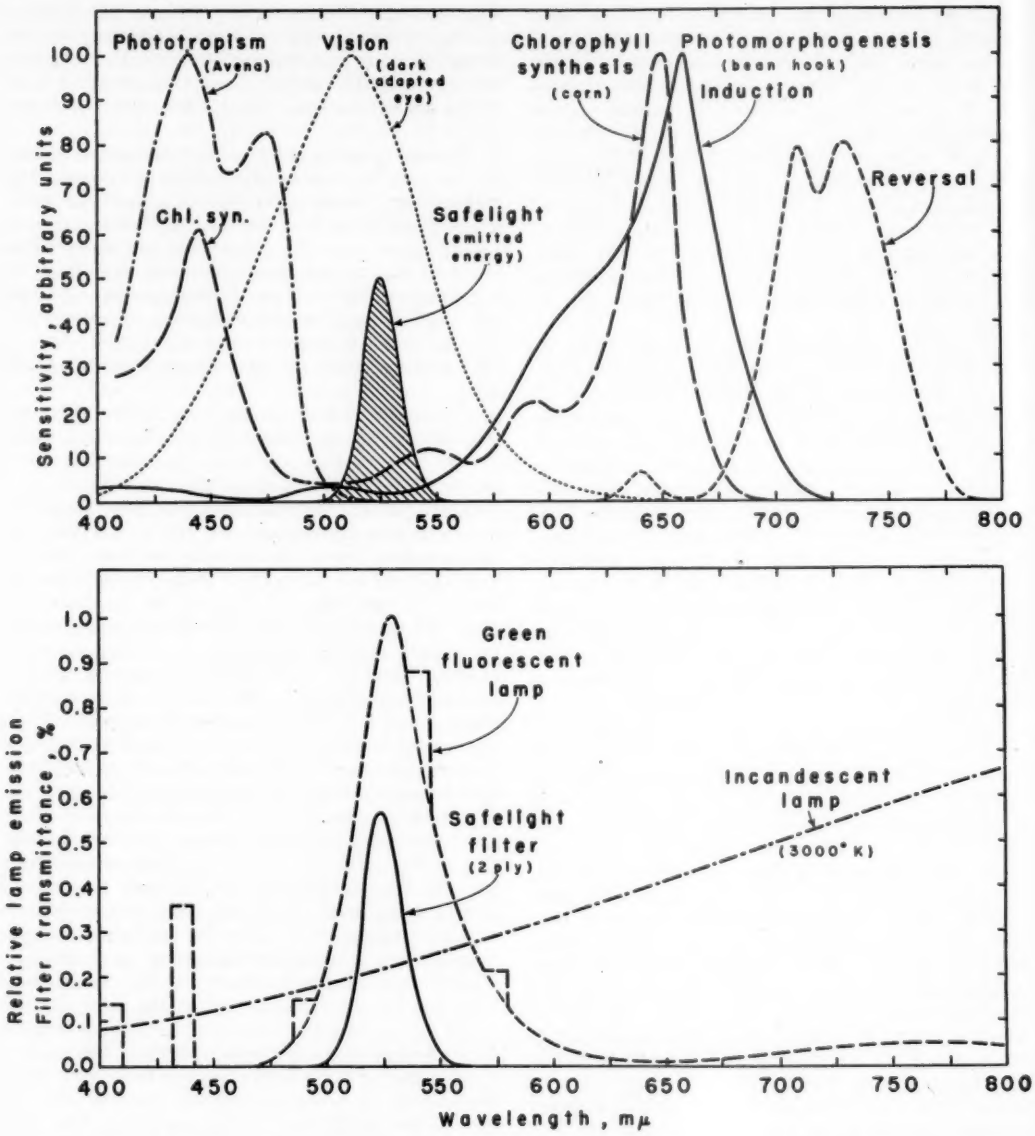

FIG. 1 (*above*). Action spectra of various biological photochemical reactions and spectral energy distribution of the safelight.

FIG. 2 (*below*). Spectral transmittance of safelight filter and relative emission of green fluorescent and incandescent lamps.

may be prepared as follows: dissolve 300 mg each of the three dyes, 8 gm sorbitol, 15 mg benzoate and 1 ml ammonium hydroxide in sufficient hot water to make a total volume of 200 ml. Cool to room temperature and add 23 gm food grade gelatin. The mixture is then heated on a water bath to about 60° C, poured onto a level glass plate and allowed to dry. We use two films in each safelight filter to eliminate possible leakage through pinholes or thin spots due to air bubbles formed during the casting.

One simple method of mounting the films is to wrap two layers around a small fluorescent lamp and exclude any emission from the ends of the tube by black tape. Films do not stand up for extended periods under these conditions, but the method is simple and they can be used for short periods in

TABLE I

PERCENT TRANSMITTANCE (T) AND COMPOSITION OF SINGLE AND DOUBLE FILMS OF THE SAFELIGHT FILTERS

WAVELENGTH, Mμ	FILTER A		FILTER B	
	1 PLY	2 PLY	1 PLY	2 PLY
320	0	0	0	0
480	0	0	0	0
490	0.1	0	0	0
500	0.8	0.006	0	0
510	3.9	0.15	0.2	0
520	7.3	0.53	4.7	0.22
525	7.5	0.56	8.8	0.78
530	6.8	0.46	13.0	1.7
540	3.8	0.14	16.0	2.6
550	1.5	0.023	14.0	2.0
560	0.2	0.004	7.9	0.62
570	0	0	3.7	0.14
580	0	0	1.0	0.01
590	0	0	0.2	0
600	0	0	0	0
760	0	0	0	0
780	0.1	0	0.1	0
800	0.2	0	0.2	0
820	0.6	0.004	0.6	0.004
840	1.5	0.02	1.5	0.02
860	3.5	0.12	3.5	0.12
880	8.5	0.72	8.5	0.72
900	19.0	3.6	19.0	3.6

Composition of one ply of a two-ply filter:

	A	B	
Dyes, duPont fabric			
Naphthol green B (C.I.* 5)	0.3	0.3	mg/cm²
Pontacyl green BL, 200 % (C.I. 666)	0.3	0.1	mg/cm²
Tartrazine (C.I. 640)	0.3	1.0	mg/cm²
Gelatin solution			
Gelatin, food grade	150		gm
Sorbitol	50		gm
Sodium benzoate	1		gm
Water	1000		ml
Rate of pouring	0.2		ml/cm²

* C.I. = Colour Index (9).

rooms at moderate humidity. Coating the film with a clear plastic, such as photographic film lacquer, is of considerable help in preventing deterioration under high humidity conditions. A second method is to mount the two filter plys between two glass panes, together with one or more layers of diffusing material, such as tracing or filter paper. The paper diffuses the light and eliminates bright areas in the background. The assembly can be taped together with black plastic electrical or photographic tape and mounted on the front of a box containing two 15-watt, 18-inch green fluorescent lamps. The box for the lamps can be made from a $3'' \times 10'' \times 23''$ standard electronic steel chassis. A convenient fluorescent fixture is the lighting strip type in which the ballast is mounted in a small narrow metal case with a lamp on the outside. Most of these units are narrow enough to fit inside a 3-inch deep electronic chassis. The open side (bottom) of the chassis is then used for mounting the filter assembly. A third method which we use is to mount the glass-enclosed safelight filter in a table top; this gives an excellent working surface for the handling of plant material. The use of two fluorescent lamps reduces the possibility of total lamp failure in the middle of an experiment.

The light intensity required for just barely seeing such materials as Avena coleoptiles and bean hooks ranges from 0.01 to 0.1 ft-c. At 0.1 ft-c with filter A, bean hooks can be exposed for about one hour without any significant hook opening response (6). An exposure to 0.1 ft-c for two hours from the B filter induced no phototropic curvature in Avena. One can work with moderate ease on a clean white surface with only 0.01 ft-c if red goggles have been worn for a half hour or more prior to working in the darkroom. Red goggles for dark-adapting the eyes are available from hospital supply houses, since this is the technic used by radiologists who need to dark-adapt their eyes for seeing the weak images of an x-ray fluorescent screen.

SUMMARY

A safelight for darkroom handling of plant material is described. During extensive tests it has been found without effect on phototropic bending of the Avena coleoptile, on photomorphogenesis in bean and pea seedlings and in chlorophyll synthesis. It has its maximum transmission in the region of maximal sensitivity of the human eye.

The safelight consists of one or more green fluorescent tubes and a dyed-gelatin filter. The composition of the filter can be varied so as to shift the transmission region either toward the blue or red, depending on the problem under investigation.

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AMERICAN SOCIETY OF PLANT PHYSIOLOGISTS, *Southern Section:* At the annual meeting in Birmingham, Alabama, February 4 to 6, 1957, the Southern Section of the American Society of Plant Physiologists elected the following officers: James L. Liverman (Texas Agricultural Experiment Station), Chairman; Aubrey W. Naylor (Duke University), Vice-Chairman; Howard J. Teas (University of Florida), Secretary-Treasurer. The directors chosen were Wayne C. Hall (Texas A. and M.), D. E. Davis (Alabama Polytech.), J. B. Whitney (Clemson College) and D. R. Ergle (U. S. Dept. Agriculture, Texas Agricultural Experiment Station). These officers took office immediately following the meeting and will serve until after next year's meeting in Little Rock, Arkansas.

We had a very outstanding meeting this year with thirteen contributed papers and a fine symposium on plant growth substances and on photoperiodism with ten papers. T. J. Marth (U. S. Dept. Agriculture, Beltsville) spoke on the effects of gibberellic acid in controlling plant growth and R. B. Withrow related experiments on the effect of light in controlling plant growth. W. N. Dannenburg gave a paper on the biosynthesis of indoleacetic acid from tryptophan and S. P. Johnson presented a paper on the effects of red and far-red light on plants growing in the southwestern United States. A joint symposium with the American Society of Agronomy concerned minor element nutrition and irrigation. Our speakers on this symposium were Howard Joham (Texas Agricultural Experiment Station), who spoke on molybdenum in the growth of cotton and W. M. Dugger, Jr. (University of Florida), who talked of his research with boron.

Our first day's session with contributed papers had about 60 people in attendance at each presentation. On the second day, the number had dwindled to between 20 and 40 depending upon the particular paper.

We had our annual breakfast on the morning of Wednesday, February 6th, at which our business meeting was held. We are planning for a big year next year at Little Rock and are already contacting members with regard to this meeting.—JAMES L. LIVERMAN, *Chairman.*

TOBACCO LITERATURE SERVICE: North Carolina State College recently announced the establishment of the world's first Tobacco Literature Service, and the publication of its first volume of *Tobacco Abstracts*, now being distributed on an international basis.

The College points out that this is a joint undertaking of the D. H. Hill Library and the Agricultural Experiment Station. It will maintain its headquarters in the Library and will be financed largely by the Experiment Station. Miss Margaret Drenowatz, a native of New Jersey and a graduate of Rutgers University Library School, has been named full-time head of the unique Tobacco Literature Service.

The Service will assemble and publish abstracts of literature on tobacco for distribution to tobacco scientists throughout the world. It also will provide library staff service at State College for any tobacco scientist.

It was pointed out that the idea for the Literature Service developed as an outgrowth of a need expressed by tobacco scientists at State College for assistance in assembling and reviewing technical literature for tobacco in the various fields of science. Dr. Ralph Shaw, Professor of Library Science at Rutgers University, President of the American Library Association, and former Director of the U. S. Department of Agriculture Library, is a consultant on this project.

The monthly issues of *Tobacco Abstracts* are being made available to libraries in the United States and in certain other countries. Those interested in subscribing to *Tobacco Abstracts* may do so by writing to the Tobacco Literature Service, D. H. Hill Library, North Carolina State College, Raleigh, North Carolina.—H. T. SCOFIELD.

TOBACCO ABSTRACTS, INTRODUCTORY ISSUE—Tobacco Literature Service, D. H. Hill Library, North Carolina State College. Agricultural Experiment Station, North Carolina State College, Raleigh, North Carolina. 32 pages. December, 1956.

Numbered abstracts and citations from world literature on Nicotiana have been culled from the following sources: Agricultural and Horticultural Engineering Abstracts, Biological Abstracts, Bibliography of Agriculture, British Abstracts of Medical Science, Battelle Technical Review, Chemical Abstracts, Helminthological Abstracts, Herbage Abstracts, Indian Tobacco, Journal of the Science of Food and Agriculture, Maryland Agricultural Journal, Plant Breeding Abstracts, and Review of Applied Mycology and have been dispersed into the following alphabetically arranged topics: bibliographies, botany, by-products, chemical and physical properties of tobacco, climatological factors, cultural practices (field), diseases, general reports and information, genetics, harvesting and curing, health, history, insects, manufacturing technology, marketing, physiology and biochemistry, policy, production economics, seedling production, soils, and varieties. An author index is provided.—ELIZABETH A. VALENTINE.

Change of Editor-in-Chief

The current volume of *Plant Physiology* (vol. 32) will be edited by the present editor, Dr. David R. Goddard. The editorial office will continue to handle current manuscripts, proofs, reprint orders, and abstracts for papers to be published in the issues for the year 1957 including the November issue. Authors with papers submitted to the journal should continue correspondence with Dr. David R. Goddard, Editor of *Plant Physiology,* Botany Department, University of Pennsylvania, Philadelphia 4, Pennsylvania until they are informed that their papers have been referred to the succeeding editor, Dr. Allan H. Brown.

The new Editor-in-Chief, Professor Allan H. Brown will handle manuscripts for publication in the January 1958 issue. Authors submitting papers after September 1, 1957 are asked to send their papers directly to Dr. Allan H. Brown, Editor of *Plant Physiology,* Department of Botany, University of Minnesota, Minneapolis 14, Minnesota.